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INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/54, 9/12, C07K 16/40, G01N 33/573, A61K 38/17, C12N 5/10

A1

(11) International Publication Number: **WO 99/09181**

(43) International Publication Date: 25 February 1999 (25.02.99)

(21) International Application Number: PCT/GB98/02475

(22) International Filing Date: 18 August 1998 (18.08.98)

(30) Priority Data:
9717416.3 19 August 1997 (19.08.97) GB

(71) Applicant *(for all designated States except US)*: MEDICAL RESEARCH COUNCIL [GB/GB]; 20 Park Crescent, London W1N 4AL (GB).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: LAWLER, Sean, Edward [GB/GB]; Aggletree Cottage, 1 South Pleasant, Settle, North Yorkshire BD24 9LA (GB). CUENDA-MENDEZ, Ana, Isabel [ES/GB]; 30 Perth Road, Dundee DD2 1LG (GB). COHEN, Philip [GB/GB]; Inverbay II, Invergowrie, Dundee DD2 5DQ (GB).

(74) Agent: THOMAS, Philip; Eric Potter Clarkson, Park View House, 58 The Ropewalk, Nottingham NG1 5DD (GB).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: HUMAN STRESS-ACTIVATED PROTEIN KINASE, SKK4

M E S I E I D Q K L Q E I M K Q T G Y L
T I G G Q R Y Q A E I N D L E N L G E M
G S G T C G Q V W K M R F R K T G H V I
A V K Q M R R S G N K E E N K R I L M D
L D V V L K S H D C P Y I V Q C F G T F
I T N T D V F I A M E L M G T C A E K L
K K R M Q G P I P E R I L G K M T V A I
V K A L Y Y L K E K H G V I H R D V K P (A)
S N I L L D E R G Q I K L C D F G I S G
R L V D S K A K T R S A G C A A Y M A P
E R I D P P D P T K P D Y D I R A D V W
S L G I S L V E L A T G Q F P Y K N C K
T D F E V L T K V L Q E E P P L L P G H
M G F S G D F Q S F V K D C L T K D H R
K R P K Y N K L L E H S F I K R Y E T L
E V D V A S W F K D V M A K T E S P R T
S G V L S Q P H L P F F R,

(57) Abstract

A substantially pure stress-activated protein kinase comprising the amino acid sequence (A) or a variant, fragment, fusion or derivative thereof, or a fusion of a said variant or fragment or derivative. The stress-activated protein kinase is useful in screening assays for drugs.

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HUMAN STRESS-ACTIVATED PROTEIN KINASE, SKK4

The present invention relates to polypeptides, polynucleotides and uses thereof, in particular to members of the stress-activated protein kinase kinase (SKK) family.

Seven mitogen-activated protein (MAP) kinase family members are activated by cellular stresses (chemical, heat and osmotic shock, ultraviolet radiation, inhibitors of protein synthesis), bacterial lipopolysaccharide (LPS), and the cytokines interleukin-1 (IL1) and tumour necrosis factor (TNF), and have therefore been termed stress-activated protein kinases or SAPKs (reviewed in Cohen, 1997; also Goedert *et al* (1997) *EMBO J* **16**, 3563-3571; Kumar *et al* (1997) *Biochem. Biophys. Res. Comm.* **235**, 533-538). The three isoforms of SAPK1 [also called c-Jun N-terminal kinases (JNKs)] phosphorylate Ser-63 and Ser-73 in the activation domain of c-Jun (Pulverer *et al*, 1991), thereby increasing its transcriptional activity. The same sites in c-Jun also become phosphorylated when cells are exposed to the stresses and cytokines that activate SAPK1 (Pulverer *et al*, 1991; Hibi *et al*, 1993; Dérijard *et al*, 1994; Kyriakis *et al*, 1994), suggesting that c-Jun is a physiological substrate for SAPK1.

SAPK2a [also termed p38 (Han *et al*, 1994), p40 (Freshney *et al*, 1994), RK (Rouse *et al*, 1994), CSBP (Lee *et al*, 1994) and Mxi2 (Zervos *et al*, 1995)] is inhibited very specifically by the pyridinyl imidazoles SB 203580 and SB 202190 (Lee *et al*, 1994; Cuenda *et al.*, 1995; reviewed in Cohen, 1997) which have been exploited to identify several physiological substrates. These include four protein kinases, namely MAP kinase-

activated protein kinase-2 (MAPKAP-K2, Rouse *et al*, 1994) and the closely related MAPKAP-K3 (McLaughlin *et al*, 1996; Ludwig *et al* (1996); Clifton *et al*, 1996), as well as MAP kinase interacting protein kinases-1 and -2 (Mnkl and Mnk2) (Waskiewicz *et al*, 1997; Fukunaga and Hunter, 1997).

Physiological substrates of MAPKAP-K2/K3 include heat shock protein (HSP) 27 (Cuenda *et al*, 1995; Huot *et al*, 1995) and the transcription factor CREB (Tan *et al*, 1996), whereas transcription factor eIF4E is a physiological substrate of Mnkl/2 (Waskiewicz *et al*, 1997). SAPK2a also mediates the stress-induced phosphorylation and activation of the CEBPβ-related transcription factor CHOP (Wang and Ron, 1996) and the ternary complex factor Elk-1 (Price *et al*, 1996).

Based on the effects of SB 203580, the activation of SAPK2a is rate-limiting in the LPS-induced production of IL1 and TNF in monocytes (Lee *et al*, 1994), in the TNF-stimulated transcription of IL6 and GM-CSF in fibroblasts (Beyaert *et al*, 1996), in the IL1-induced stimulation of glucose uptake in epithelial cells (Gould *et al*, 1995), in collagen-induced platelet aggregation (Saklatvala *et al*, 1996) and in the stress-induced transcription of c-Jun and c-Fos in fibroblasts (Hazzalin *et al*, 1996; Price *et al*, 1996). The SAPK2a catalysed phosphorylation of Elk-1 (Price *et al*, 1996) and the MAPKAP-K2 catalysed phosphorylation of CREB (Tan *et al*, 1996) are both likely to contribute to the stress-induced transcription of c-fos (Ginty *et al*, 1994).

Recently, two additional SAP kinases were identified, called SAPK2b [or p38β (Jiang *et al*, 1996)] and SAPK3 (Mertens *et al*, 1996) [also called

ERK6 (Lechner *et al*, 1996) and p38γ (Li *et al*, 1996)]. The amino acid sequence of SAPK2b is 73% identical to SAPK2a and it is inhibited by SB 202190 and SB 203580 at similar concentrations to SAPK2a. In contrast, the amino acid sequence of SAPK3 is only 60% identical to SAPK2a and SAPK3 is not inhibited by SB 203580 (Cuenda *et al*, 1997). SAPK2b and SAPK3 have been introduced into mammalian cells by transient transfection and shown to be activated in response to pro-inflammatory cytokines and stressful stimuli in a manner similar to SAPK1 and SAPK2a. The physiological roles of SAPK2b and SAPK3 are unknown. The mRNAs encoding these enzymes are present in all mammalian tissues examined (Jiang *et al*, 1996; Mertens *et al*, 1996; Goedert et al., 1997), with the mRNA encoding SAPK3 being highest in skeletal muscle. Expression of wild-type SAPK3 and an inactive mutant in the muscle cell line C2C12 enhanced and inhibited differentiation into myotubes, respectively (Lechner *et al*, 1996). *In vitro*, SAPK2b and SAPK3 phosphorylated several proteins that are also substrates for SAPK2a. SAPK2b was reported to phosphorylate the transcription factor ATF2 more efficiently than SAPK2a (Jiang *et al.*, 1996) but, since the stress- and cytokine- induced phosphorylation of ATF2 in fibroblasts is unaffected by SB 203580 (Hazzalin *et al.*, 1996; Beyaert et al., 1996), neither SAPK2a nor SAPK2b appears to be rate-limiting for ATF2 phosphorylation *in vivo*, in contrast to earlier studies using transfection-based approaches (Gupta *et al*, 1995). The substrate specificity of SAPK3 *in vitro* was similar to that of SAPK2a, except that it was much less effective in activating MAPKAP-K2/K3 and (like SAPK1, but unlike SAPK2a) phosphorylated ATF2 at Ser9O, as well as at Thr69 and Thr71 (Cuenda *et al*, 1997). However, whether SAPK1 and/or SAPK3 are rate-limiting for ATF2 phosphorylation *in vivo* is unknown.

SAPK4 is the seventh MAP kinase family member. This enzyme, which also contains a TGY sequence in the activation domain, shows about 60% identity to SAPK2a, SAPK2b and SAPK3 and its mRNA is widely expressed in human tissues. SAPK4 is activated by the same stimuli that activate other SAP kinases, has an *in vitro* substrate specificity similar to that of SAPK3 and, like SAPK3, is not inhibited by SB 203480 or SB 202190.

Five chromatographically distinct SAP kinase kinases (SAPKKs or SKKs) have been identified in mammalian cells (Meier *et al*, 1996; Cuenda *et al*, 1996). *In vitro*, SKK1 [also termed MKK4 (Dérijard *et al*, 1995), SEK1 (Sanchez *et al*, 1994) and XMEK2 (Yashar *et al*, 1993)] activates all four SAPKs (Sanchez *et al*, 1994; Dérijard *et al*, 1995; Doza *et al*, 1995; Jiang *et al*, 1996; Cuenda *et al*, 1997), although SAPK2b and SAPK3 are phosphorylated less efficiently. SKK2 [also termed MKK3 (Dérijard *et al*, 1995)] and SKK3 (Cuenda *et al*, 1996) [also called MKK6 (Han *et al*, 1996; Moriguchi *et al*, 1996; Raingeaud *et al*, 1996) and MEK6 (Stein *et al*, 1996)] activate SAPK2a but not SAPK1, while SKK3 was the only detectable activator of SAPK3 induced by pro-inflammatory cytokines and stressful stimuli in human epithelial KB cells or human embryonic kidney 293 cells (Cuenda *et al*, 1997). SKK3 was also the most efficient activator of SAPK2b in co-transfection experiments (Jiang *et al*, 1996), and was the only detectable activator of SAPK4 induced by pro-inflammatory cytokines and stressful stimuli in KB cells [Goedert *et al* (1997) *EMBO J* **16**, 3563-3571]. SKK4 and SKK5 activate SAPK1 but not SAPK2a (Meier *et al*, 1996) or SAPK3 (Cuenda *et al*, 1997).

The cloning of a mouse MAP kinase kinase family member termed MKK7 has recently been reported in Tournier *et al* (1997) *PNAS* **94**, 7337-7342.

The importance of different SKKs in activating SAPK1 was not previously resolved. SKK1/MKK4 is the only activator of SAPK1/JNK that can be detected biochemically in extracts prepared from PC12 cells that have been exposed to adverse stimuli (Meier *et al* (1996)), and in 293 cells the activation of transfected SAPK1/JNK by anisomycin can be prevented by overexpressing a catalytically inactive form of SKK1/MKK4 (Sanchez *et al* (1994) *Nature* **372**, 794-798). In addition, heat shock or anisomycin failed to activate SAPK1/JNK in murine embryonic stem cells lacking SKK1/MKK4 (Nishina *et al* (1997) *Nature* **385**, 350-353, Yang *et al* (1997) *PNAS* **94**, 3004-3009). SAPK1/JNK is activated strongly by cotransfection with MEK kinase (MEKK) (Yan *et al* (1994) *Nature* **372**, 798-800). MEK Kinase is an upstream activator of SKK1 that is activated by osmotic shock in rat 3Y1 fibroblasts and PC12 cells (Yan *et al* (1994) *Nature* **372**, 798-800; Matsuda *et al* (1995) *J. Biol. Chem.* **270**, 12781-12786)).

Although SKK1/MKK4 is clearly essential for the activation of SAPK1/JNK in some cells, the dominant activator of SAPK1/JNK detected biochemically in human epithelial KB cells (Meier *et al* (1996) or in Rat 3Y1 fibroblasts (Moriguchi *et al* (1995) is a chromatographically distinct enzyme(s). In Meier *et al,* (1996), SKK4 was partially resolved from SKK3 by elution from Mono S using a sodium chloride gradient, but purification of SKK4 was not achieved. Moriguchi *et al,* (1995) identified multiple SAP kinase kinases using a method employing Q-sepharose and

heparin-sepharose chromatography. This enabled the presence of a kinase that may correspond to SKK4 as identified in Meier *et al,* (1996) to be detected, but purification of the kinase was not reported.

Moreover, the activation of SAPK1/JNK by UV radiation and osmotic shock in murine embryonic stem cells lacking SKK1/MKK4 was either unimpaired (Nishina *et al* (1997)) or only inhibited partially (Yang *et al* (1997)). These observations indicate that mammalian cells contain at least one further activator of SAPK1/JNK distinct from SKK1/MKK4.

We here report the cloning of a novel SAP kinase kinase family member that we call SKK4. The mRNA is widely expressed in human tissues. The amino acid sequence of this enzyme is most similar (62.4% identity) to a fruit fly (*Drosophila*) MKK homologue (HEP) which geneticists have shown to lie upstream of JNK in a pathway essential for embryonic development of the fruit fly. It is also related to the human SKK1, SKK2 and SKK3 enzymes, with 47.7%, 41.4% and 39.3% amino acid sequence identity respectively. It has 36% amino acid sequence identity with MKK1, and 35% with MKK2.

We report that SKK4 activates SAPK1/JNK *in vitro*, but not SAPK2a/p38, SAPK3/ERK6 or SAPK4. In human epithelial KB cells SKK4 and SKK1/MKK4 (another activator of SAPK1/JNK) are both activated by stressful stimuli, but only SKK4 is activated strongly by proinflammatory cytokines. Recombinant SKK4 can be activated by incubation with MgATP and MEKK. The identification of SKK4 explains why the major SAPK1/JNK activator detected in many mammalian cell extracts is chromatographically separable from SKK1/MKK4.

SKK4 may be a more attractive target for an anti-inflammatory drug than SAPK1/JNK because inhibitors of SKK4 may prevent the activation of SAPK1/JNK by proinflammatory cytokines, without affecting the activation of SAPK1/JNK by other agents.

A first aspect of the invention provides a substantially pure polypeptide comprising the amino acid sequence

M E S I E I D Q K L Q E I M K Q T G Y L
T I G G Q R Y Q A E I N D L E N L G E M
G S G T C G Q V W K M R F R K T G H V I
A V K Q M R R S G N K E E N K R I L M D
L D V V L K S H D C P Y I V Q C F G T F
I T N T D V F I A M E L M G T C A E K L
K K R M Q G P I P E R I L G K M T V A I
V K A L Y Y L K E K H G V I H R D V K P
S N I L L D E R G Q I K L C D F G I S G
R L V D S K A K T R S A G C A A Y M A P
E R I D P P D P T K P D Y D I R A D V W
S L G I S L V E L A T G Q F P Y K N C K
T D F E V L T K V L Q E E P P L L P G H
M G F S G D F Q S F V K D C L T K D H R
K R P K Y N K L L E H S F I K R Y E T L
E V D V A S W F K D V M A K T E S P R T
S G V L S Q P H L P F F R,

or a variant, fragment, fusion or derivative thereof, or a fusion of a said variant or fragment or derivative. This polypeptide is considered to be a stress-activated protein kinase kinase.

The polypeptide with the amino acid sequence as shown above is herein referred to as SKK4 (stress-activated protein kinase kinase 4).

By "substantially pure" we mean that the said polypeptide is substantially free of other proteins. Thus, we include any composition that includes at least 30% of the protein content by weight as the said polypeptide, preferably at least 50%, more preferably at least 70%, still more preferably at least 90% and most preferably at least 95% of the protein content is the said polypeptide.

Thus, the invention also includes compositions comprising the said polypeptide and a contaminant wherein the contaminant comprises less than 70% of the composition by weight, preferably less than 50% of the composition, more preferably less than 30% of the composition, still more preferably less than 10% of the composition and most preferably less than 5% of the composition by weight.

The invention also includes the substantially pure said polypeptide when combined with other components *ex vivo*, said other components not being all of the components found in the cell in which said polypeptide is found.

Variants (whether naturally-occurring or otherwise) may be made using the methods of protein engineering and site-directed mutagenesis well known in the art using the recombinant polynucleotides described below.

By "fragment of said polypeptide" we include any fragment which retains activity or which is useful in some other way, for example, for use in raising antibodies or in a binding assay.

By "fusion of said polypeptide" we include said polypeptide fused to any other polypeptide. For example, the said polypeptide may be fused to a polypeptide such as glutathione-S-transferase (GST) or protein A in order to facilitate purification of said polypeptide. Similarly, the said polypeptide may be fused to an oligo-histidine tag such as His_6 or to an epitope recognised by an antibody such as the well known Myc tag epitope. Fusions to any variant, fragment or derivative of said polypeptide are also included in the scope of the invention.

By "variants" of the polypeptide we include insertions, deletions and substitutions, either conservative or non-conservative. In particular we include variants of the polypeptide where such changes do not substantially alter the activity of the said polypeptide. Variants of SKK4 do not include polypeptides which have the amino acid sequence of human SKK1, SKK2, SKK3 or *D. melanogaster* HEP.

By "conservative substitutions" is intended combinations such as Gly, Ala; Val, Ile, Leu; Asp, Glu; Asn, Gln; Ser, Thr; Lys, Arg; and Phe, Tyr.

It is particularly preferred if the polypeptide variant has an amino acid sequence which has at least 98.8% identity with the amino acid sequence given above, more preferably at least 99.1%, still more preferably at least 99.4%, yet more preferably at least 99.7%, and most preferably at least 99.9% identity with the amino acid sequence given above.

The percent sequence identity between two polypeptides may be determined using suitable computer programs, for example the GAP program of the University of Wisconsin Genetic Computing Group and it

will be appreciated that percent identity is calculated in relation to polypeptides whose sequences have been aligned optimally.

A particular embodiment of the invention provides a substantially pure human SKK4 polypeptide which consists of the amino acid sequence

M E S I E I D Q K L Q E I M K Q T G Y L
T I G G Q R Y Q A E I N D L E N L G E M
G S G T C G Q V W K M R F R K T G H V I
A V K Q M R R S G N K E E N K R I L M D
L D V V L K S H D C P Y I V Q C F G T F
I T N T D V F I A M E L M G T C A E K L
K K R M Q G P I P E R I L G K M T V A I
V K A L Y Y L K E K H G V I H R D V K P
S N I L L D E R G Q I K L C D F G I S G
R L V D S K A K T R S A G C A A Y M A P
E R I D P P D P T K P D Y D I R A D V W
S L G I S L V E L A T G Q F P Y K N C K
T D F E V L T K V L Q E E P P L L P G H
M G F S G D F Q S F V K D C L T K D H R
K R P K Y N K L L E H S F I K R Y E T L
E V D V A S W F K D V M A K T E S P R T
S G V L S Q P H L P F F R,

or naturally occurring allelic variants thereof. The amino acid sequence is also shown as the translation of a polynucleotide sequence in Figure 1.

It is particularly preferred, although not essential, that the variant or fragment or derivative or fusion of the said polypeptide, or the fusion of the variant or fragment or derivative has at least 30% of the enzyme activity of SKK4 with respect to the phosphorylation of SAPK1. It is more preferred if the variant or fragment or derivative or fusion of the said polypeptide, or the fusion of the variant or fragment or derivative has at least 50%, preferably at least 70% and more preferably at least 90% of

the enzyme activity of SKK4 with respect to the phosphorylation of SAPK1. However, it will be appreciated that variants or fusions or derivatives or fragments which are devoid of enzymatic activity may nevertheless be useful, for example by interacting with another polypeptide, or as antigens in raising antibodies.

A second aspect of the invention provides a recombinant polynucleotide encoding a polypeptide as defined in the first aspect of the invention or encoding a variant or fragment or derivative of fusion of said polypeptide or a fusion of a said variant or fragment or derivative. Preferences and exclusions for the said polynucleotide variant are the same as in the first aspect of the invention, except that the following Expressed Sequence Tags (ESTs) are also excluded:

AA256025, AA019720, AA252650, H85962, AA194205, W58120, (all human)
AA451434, AA194047 (mouse)

In one preferred embodiment the polynucleotide comprises the nucleotide sequence

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ATGGAGAGCATTGAGATTGACCAGAAGCTGCAGGAGATCATGAAGCAGACGGGCTACC
TGACCATCGGGGGCCAGCGCTACCAGGCAGAAATCAACGACCTGGAGAACTTGGGCGA
GATGGGCAGCGGCACCTGCGGCCAGGTGTGGAAGATGCGCTTCCGGAAGACCGGCCAC
GTCATTGCCGTTAAGCAAATGCGGCGCTCCGGGAACAAGGAGGAGAACAAGCGCATCC
TCATGGACCTGGATGTGGTGCTGAAGAGCCACGACTGCCCCTACATCGTGCAGTGCTT
TGGGACGTTCATCACCAACACGGACGTCTTCATCGCCATGGAGCTCATGGGCACCTGC
GCTGAGAAGCTCAAGAAGCGGATGCAGGGCCCCATCCCCGAGCGCATTCTGGGCAAGA
TGACAGTGGCGATTGTGAAGGCGCTGTACTACCTGAAGGAGAAGCACGGTGTCATCCA
CCGCGACGTCAAGCCCTCCAACATCCTGCTGGACGAGCGGGGCCAGATCAAGCTCTGC
GACTTCGGCATCAGCGGCCGCCTGGTGGACTCCAAAGCCAAGACGCGGAGCGCCGGCT
GTGCCGCCTACATGGCACCCGAGCGCATTGACCCCCCAGACCCCACCAAGCCGGACTA
TGACATCCGGGCCGACGTATGGAGCCTGGGCATCTCGTTGGTGGAGCTGGCAACAGGA
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CAGTTTCCCTACAAGAACTGCAAGACGGACTTTGAGGTCCTCACCAAAGTCCTACAGG
AAGAGCCCCCGCTTCTGCCCGGACACATGGGCTTCTCGGGGGACTTCCAGTCCTTCGT
CAAAGACTGCCTTACTAAAGATCACAGGAAGAGACCAAAGTATAATAAGCTACTTGAA
CACAGCTTCATCAAGCGCTACGAGACGCTGGAGGTGGACGTGGCGTCCTGGTTCAAGG
ATGTCATGGCGAAGACTGAGTCACCGCGGACTAGCGGCGTCCTGAGCCAGCCCCACCT
GCCCTTCTTCAGG,

or a variant, fragment, fusion or derivative thereof. The nucleotide sequence is shown in Figure 1 together with the translation of the relevant open reading frame.

The invention also includes a polynucleotide comprising a fragment of the recombinant polynucleotide of the second aspect of the invention. Preferably, the polynucleotide comprises a fragment which is at least 10 nucleotides in length, more preferably at least 14 nucleotides in length and still more preferably at least 18 nucleotides in length. Such polynucleotides are useful as PCR primers.

The polynucleotide or recombinant polynucleotide may be DNA or RNA, preferably DNA. The polynucleotide may or may not contain introns in the coding sequence; preferably the polynucleotide is a cDNA.

A "variation" of the polynucleotide includes one which is (i) usable to produce a protein or a fragment thereof which is in turn usable to prepare antibodies which specifically bind to the protein encoded by the said polynucleotide or (ii) an antisense sequence corresponding to the gene or to a variation of type (i) as just defined. For example, different codons can be substituted which code for the same amino acid(s) as the original codons. Alternatively, the substitute codons may code for a different

amino acid that will not affect the activity or immunogenicity of the protein or which may improve or otherwise modulate its activity or immunogenicity. For example, site-directed mutagenesis or other techniques can be employed to create single or multiple mutations, such as replacements, insertions, deletions, and transpositions, as described in Botstein and Shortle, "Strategies and Applications of *In Vitro* Mutagenesis," *Science*, **229:** 193-210 (1985), which is incorporated herein by reference. Since such modified polynucleotides can be obtained by the application of known techniques to the teachings contained herein, such modified polynucleotides are within the scope of the claimed invention.

Moreover, it will be recognised by those skilled in the art that the polynucleotide sequence (or fragments thereof) of the invention can be used to obtain other polynucleotide sequences that hybridise with it under conditions of high stringency. Such polynucleotides includes any genomic DNA. Accordingly, the polynucleotide of the invention includes polynucleotide that shows at least 89.5%, preferably 92%, and more preferably at least 95% and most preferably at least 99% homology with the polynucleotide identified in the method of the invention, provided that such homologous polynucleotide encodes a polypeptide which is usable in at least some of the methods described below or is otherwise useful.

Per cent homology can be determined by, for example, the GAP program of the University of Wisconsin Genetic Computer Group.

DNA-DNA, DNA-RNA and RNA-RNA hybridisation may be performed in aqueous solution containing between 0.1XSSC and 6XSSC and at

temperatures of between 55°C and 70°C. It is well known in the art that the higher the temperature or the lower the SSC concentration the more stringent the hybridisation conditions. By "high stringency" we mean 2XSSC and 65°C. 1XSSC is 0.15M NaCl/0.015M sodium citrate. Polynucleotides which hybridise at high stringency are included within the scope of the claimed invention.

"Variations" of the polynucleotide also include polynucleotide in which relatively short stretches (for example 20 to 50 nucleotides) have a high degree of homology (at least 95% and preferably at least 99 or 99.9%) with equivalent stretches of the polynucleotide of the invention even though the overall homology between the two polynucleotides may be much less. This is because important active or binding sites may be shared even when the general architecture of the protein is different.

A further aspect of the invention provides a replicable vector comprising a recombinant polynucleotide encoding a said polypeptide or a variant, fragment, derivative or fusion of said polypeptide or a fusion of said variant, fragment or derivative.

A variety of methods have been developed to operably link polynucleotides, especially DNA, to vectors for example *via* complementary cohesive termini. For instance, complementary homopolymer tracts can be added to the DNA segment to be inserted to the vector DNA. The vector and DNA segment are then joined by hydrogen bonding between the complementary homopolymeric tails to form recombinant DNA molecules.

Synthetic linkers containing one or more restriction sites provide an alternative method of joining the DNA segment to vectors. The DNA segment, generated by endonuclease restriction digestion as described earlier, is treated with bacteriophage T4 DNA polymerase or *E. coli* DNA polymerase I, enzymes that remove protruding, 3'-single-stranded termini with their 3'-5'-exonucleolytic activities, and fill in recessed 3'-ends with their polymerizing activities.

The combination of these activities therefore generates blunt-ended DNA segments. The blunt-ended segments are then incubated with a large molar excess of linker molecules in the presence of an enzyme that is able to catalyze the ligation of blunt-ended DNA molecules, such as bacteriophage T4 DNA ligase. Thus, the products of the reaction are DNA segments carrying polymeric linker sequences at their ends. These DNA segments are then cleaved with the appropriate restriction enzyme and ligated to an expression vector that has been cleaved with an enzyme that produces termini compatible with those of the DNA segment.

Synthetic linkers containing a variety of restriction endonuclease sites are commercially available from a number of sources including International Biotechnologies Inc, New Haven, CN, USA.

A desirable way to modify the DNA encoding a polypeptide of the invention is to use the polymerase chain reaction as disclosed by Saiki *et al* (1988) *Science* **239**, 487-491. This method may be used for introducing the DNA into a suitable vector, for example by engineering in suitable restriction sites, or it may be used to modify the DNA in other useful ways as is known in the art.

In this method the DNA to be enzymatically amplified is flanked by two specific primers which themselves become incorporated into the amplified DNA. The said specific primers may contain restriction endonuclease recognition sites which can be used for cloning into expression vectors using methods known in the art.

The DNA (or in the case of retroviral vectors, RNA) is then expressed in a suitable host to produce a polypeptide comprising the compound of the invention. Thus, the DNA encoding the polypeptide constituting the compound of the invention may be used in accordance with known techniques, appropriately modified in view of the teachings contained herein, to construct an expression vector, which is then used to transform an appropriate host cell for the expression and production of the polypeptide of the invention. Such techniques include those disclosed in US Patent Nos. 4,440,859 issued 3 April 1984 to Rutter *et al*, 4,530,901 issued 23 July 1985 to Weissman, 4,582,800 issued 15 April 1986 to Crowl, 4,677,063 issued 30 June 1987 to Mark *et al*, 4,678,751 issued 7 July 1987 to Goeddel, 4,704,362 issued 3 November 1987 to Itakura *et al*, 4,710,463 issued 1 December 1987 to Murray, 4,757,006 issued 12 July 1988 to Toole, Jr. *et al*, 4,766,075 issued 23 August 1988 to Goeddel *et al* and 4,810,648 issued 7 March 1989 to Stalker, all of which are incorporated herein by reference.

The DNA (or in the case of retroviral vectors, RNA) encoding the polypeptide constituting the compound of the invention may be joined to a wide variety of other DNA sequences for introduction into an appropriate host. The companion DNA will depend upon the nature of the host, the

manner of the introduction of the DNA into the host, and whether episomal maintenance or integration is desired.

Generally, the DNA is inserted into an expression vector, such as a plasmid, in proper orientation and correct reading frame for expression. If necessary, the DNA may be linked to the appropriate transcriptional and translational regulatory control nucleotide sequences recognised by the desired host, although such controls are generally available in the expression vector. The vector is then introduced into the host through standard techniques. Generally, not all of the hosts will be transformed by the vector. Therefore, it will be necessary to select for transformed host cells. One selection technique involves incorporating into the expression vector a DNA sequence, with any necessary control elements, that codes for a selectable trait in the transformed cell, such as antibiotic resistance. Alternatively, the gene for such selectable trait can be on another vector, which is used to co-transform the desired host cell.

Host cells that have been transformed by the recombinant DNA of the invention are then cultured for a sufficient time and under appropriate conditions known to those skilled in the art in view of the teachings disclosed herein to permit the expression of the polypeptide, which can then be recovered.

Many expression systems are known, including bacteria (for example *E. coli* and *Bacillus subtilis*), yeasts (for example *Saccharomyces cerevisiae*), filamentous fungi (for example *Aspergillus*), plant cells, animal cells and insect cells.

The vectors include a prokaryotic replicon, such as the ColE1 *ori*, for propagation in a prokaryote, even if the vector is to be used for expression in other, non-prokaryotic, cell types. The vectors can also include an appropriate promoter such as a prokaryotic promoter capable of directing the expression (transcription and translation) of the genes in a bacterial host cell, such as *E. coli*, transformed therewith.

A promoter is an expression control element formed by a DNA sequence that permits binding of RNA polymerase and transcription to occur. Promoter sequences compatible with exemplary bacterial hosts are typically provided in plasmid vectors containing convenient restriction sites for insertion of a DNA segment of the present invention.

Typical prokaryotic vector plasmids are pUC18, pUC19, pBR322 and pBR329 available from Biorad Laboratories, (Richmond, CA, USA) and p*Trc*99A and pKK223-3 available from Pharmacia, Piscataway, NJ, USA.

A typical mammalian cell vector plasmid is pSVL available from Pharmacia, Piscataway, NJ, USA. This vector uses the SV40 late promoter to drive expression of cloned genes, the highest level of expression being found in T antigen-producing cells, such as COS-1 cells.

An example of an inducible mammalian expression vector is pMSG, also available from Pharmacia. This vector uses the glucocorticoid-inducible promoter of the mouse mammary tumour virus long terminal repeat to drive expression of the cloned gene.

Useful yeast plasmid vectors are pRS403-406 and pRS413-416 and are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Plasmids pRS403, pRS404, pRS405 and pRS406 are Yeast Integrating plasmids (YIps) and incorporate the yeast selectable markers *HIS3*, *TRP1*, *LEU2* and *URA3*. Plasmids pRS413-416 are Yeast Centromere plasmids (YCps).

The present invention also relates to a host cell transformed with a polynucleotide vector construct of the present invention. The host cell can be either prokaryotic or eukaryotic. Bacterial cells are preferred prokaryotic host cells and typically are a strain of *E. coli* such as, for example, the *E. coli* strains DH5 available from Bethesda Research Laboratories Inc., Bethesda, MD, USA, and RR1 available from the American Type Culture Collection (ATCC) of Rockville, MD, USA (No ATCC 31343). Preferred eukaryotic host cells include yeast, insect and mammalian cells, preferably vertebrate cells such as those from a mouse, rat, monkey or human fibroblastic cell line. Yeast host cells include YPH499, YPH500 and YPH501 which are generally available from Stratagene Cloning Systems, La Jolla, CA 92037, USA. Preferred mammalian host cells include Chinese hamster ovary (CHO) cells available from the ATCC as CCL61, NIH Swiss mouse embryo cells NIH/3T3 available from the ATCC as CRL 1658, and monkey kidney-derived COS-1 cells available from the ATCC as CRL 1650. Preferred insect cells are Sf9 cells which can be transfected with baculovirus expression vectors.

Transformation of appropriate cell hosts with a DNA construct of the present invention is accomplished by well known methods that typically

depend on the type of vector used. With regard to transformation of prokaryotic host cells, see, for example, Cohen *et al* (1972) *Proc. Natl. Acad. Sci. USA* **69**, 2110 and Sambrook *et al* (1989) *Molecular Cloning, A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. Transformation of yeast cells is described in Sherman *et al* (1986) *Methods In Yeast Genetics, A Laboratory Manual*, Cold Spring Harbor, NY. The method of Beggs (1978) *Nature* **275**, 104-109 is also useful. With regard to vertebrate cells, reagents useful in transfecting such cells, for example calcium phosphate and DEAE-dextran or liposome formulations, are available from Stratagene Cloning Systems, or Life Technologies Inc., Gaithersburg, MD 20877, USA.

Electroporation is also useful for transforming and/or transfecting cells and is well known in the art for transforming yeast cell, bacterial cells, insect cells and vertebrate cells.

For example, many bacterial species may be transformed by the methods described in Luchansky *et al* (1988) *Mol. Microbiol.* 2, 637-646 incorporated herein by reference. The greatest number of transformants is consistently recovered following electroporation of the DNA-cell mixture suspended in 2.5X PEB using 6250V per cm at 25μFD.

Methods for transformation of yeast by electroporation are disclosed in Becker & Guarente (1990) *Methods Enzymol.* **194**, 182.

Successfully transformed cells, ie cells that contain a DNA construct of the present invention, can be identified by well known techniques. For example, cells resulting from the introduction of an expression construct

of the present invention can be grown to produce the polypeptide of the invention. Cells can be harvested and lysed and their DNA content examined for the presence of the DNA using a method such as that described by Southern (1975) *J. Mol. Biol.* **98**, 503 or Berent *et al* (1985) *Biotech.* 3, 208. Alternatively, the presence of the protein in the supernatant can be detected using antibodies as described below.

In addition to directly assaying for the presence of recombinant DNA, successful transformation can be confirmed by well known immunological methods when the recombinant DNA is capable of directing the expression of the protein. For example, cells successfully transformed with an expression vector produce proteins displaying appropriate antigenicity. Samples of cells suspected of being transformed are harvested and assayed for the protein using suitable antibodies.

Thus, in addition to the transformed host cells themselves, the present invention also contemplates a culture of those cells, preferably a monoclonal (clonally homogeneous) culture, or a culture derived from a monoclonal culture, in a nutrient medium.

A further aspect of the invention provides a method of making the polypeptide of the invention or a variant, derivative, fragment or fusion thereof or a fusion of a variant, fragment or derivative the method comprising culturing a host cell comprising a recombinant polynucleotide or a replicable vector which encodes said polypeptide, and isolating said polypeptide or a variant, derivative, fragment or fusion thereof or a fusion of a variant, fragment or derivative from said host cell. Methods of

cultivating host cells and isolating recombinant proteins are well known in the art.

The invention also includes a polypeptide, or a variant, fragment, derivative or fusion thereof, or fusion of a said variant or fragment or derivative obtainable by the above method of the invention.

A still further aspect of the invention provides an antibody reactive towards a polypeptide of the invention. Examples of such antibodies are given in Example 1.

Antibodies reactive towards the said polypeptide of the invention may be made by methods well known in the art. In particular, the antibodies may be polyclonal or monoclonal.

Suitable monoclonal antibodies which are reactive towards the said polypeptide may be prepared by known techniques, for example those disclosed in "*Monoclonal Antibodies: A manual of techniques*", H Zola (CRC Press, 1988) and in "Monoclonal Hybridoma Antibodies: Techniques and Applications", SGR Hurrell (CRC Press, 1982).

In a preferred embodiment the antibody is raised using any suitable peptide sequence obtainable from the given amino acid sequence of SKK4. It is preferred if polyclonal antipeptide antibodies are made.

It is particularly preferred if the antibody does not react substantially with another stress-activated protein kinase kinase such as SKK1, SKK2 or SKK3. Accordingly, it may be preferred if peptides based on the SKK4

sequence are used which vary significantly from any peptides found in any other stress-activated protein kinases such as SKK1, SKK2 or SKK3.

Peptides in which one or more of the amino acid residues are chemically modified, before or after the peptide is synthesised, may be used providing that the function of the peptide, namely the production of specific antibodies *in vivo*, remains substantially unchanged. Such modifications include forming salts with acids or bases, especially physiologically acceptable organic or inorganic acids and bases, forming an ester or amide of a terminal carboxyl group, and attaching amino acid protecting groups such as N-t-butoxycarbonyl. Such modifications may protect the peptide from *in vivo* metabolism. The peptides may be present as single copies or as multiples, for example tandem repeats. Such tandem or multiple repeats may be sufficiently antigenic themselves to obviate the use of a carrier. It may be advantageous for the peptide to be formed as a loop, with the N-terminal and C-terminal ends joined together, or to add one or more Cys residues to an end to increase antigenicity and/or to allow disulphide bonds to be formed. If the peptide is covalently linked to a carrier, preferably a polypeptide, then the arrangement is preferably such that the peptide of the invention forms a loop.

According to current immunological theories, a carrier function should be present in any immunogenic formulation in order to stimulate, or enhance stimulation of, the immune system. It is thought that the best carriers embody (or, together with the antigen, create) a T-cell epitope. The peptides may be associated, for example by cross-linking, with a separate carrier, such as serum albumins, myoglobins, bacterial toxoids and

keyhole limpet haemocyanin. More recently developed carriers which induce T-cell help in the immune response include the hepatitis-B core antigen (also called the nucleocapsid protein), presumed T-cell epitopes such as Thr-Ala-Ser-Gly-Val-Ala-Glu-Thr-Thr-Asn-Cys, beta-galactosidase and the 163-171 peptide of interleukin-1. The latter compound may variously be regarded as a carrier or as an adjuvant or as both. Alternatively, several copies of the same or different peptides of the invention may be cross-linked to one another; in this situation there is no separate carrier as such, but a carrier function may be provided by such cross-linking. Suitable cross-linking agents include those listed as such in the Sigma and Pierce catalogues, for example glutaraldehyde, carbodiimide and succinimidyl 4-(N-maleimidomethyl)cyclohexane-1-carboxylate, the latter agent exploiting the -SH group on the C-terminal cysteine residue (if present).

If the peptide is prepared by expression of a suitable nucleotide sequence in a suitable host, then it may be advantageous to express the peptide as a fusion product with a peptide sequence which acts as a carrier. Kabigen's "Ecosec" system is an example of such an arrangement.

The peptide of the invention may be linked to other antigens to provide a dual effect.

Peptides may be synthesised by the Fmoc-polyamide mode of solid-phase peptide synthesis as disclosed by Lu *et al* (1981) *J. Org. Chem.* **46**, 3433 and references therein. Temporary N-amino group protection is afforded by the 9-fluorenylmethyloxycarbonyl (Fmoc) group. Repetitive cleavage of this highly base-labile protecting group is effected using 20% piperidine

in N,N-dimethylformamide. Side-chain functionalities may be protected as their butyl ethers (in the case of serine threonine and tyrosine), butyl esters (in the case of glutamic acid and aspartic acid), butyloxycarbonyl derivative (in the case of lysine and histidine), trityl derivative (in the case of cysteine) and 4-methoxy-2,3,6-trimethylbenzenesulphonyl derivative (in the case of arginine). Where glutamine or asparagine are C-terminal residues, use is made of the 4,4'-dimethoxybenzhydryl group for protection of the side chain amido functionalities. The solid-phase support is based on a polydimethyl-acrylamide polymer constituted from the three monomers dimethylacrylamide (backbone-monomer), bisacryloylethylene diamine (cross linker) and acryloylsarcosine methyl ester (functionalising agent). The peptide-to-resin cleavable linked agent used is the acid-labile 4-hydroxymethyl-phenoxyacetic acid derivative. All amino acid derivatives are added as their preformed symmetrical anhydride derivatives with the exception of asparagine and glutamine, which are added using a reversed N,N-dicyclohexyl-carbodiimide/1-hydroxybenzotriazole mediated coupling procedure. All coupling and deprotection reactions are monitored using ninhydrin, trinitrobenzene sulphonic acid or isotin test procedures. Upon completion of synthesis, peptides are cleaved from the resin support with concomitant removal of side-chain protecting groups by treatment with 95% trifluoroacetic acid containing a 50% scavenger mix. Scavengers commonly used are ethanedithiol, phenol, anisole and water, the exact choice depending on the constituent amino acids of the peptide being synthesised. Trifluoroacetic acid is removed by evaporation *in vacuo*, with subsequent trituration with diethyl ether affording the crude peptide. Any scavengers present are removed by a simple extraction procedure which on lyophilisation of the aqueous phase affords the crude peptide free of

scavengers. Reagents for peptide synthesis are generally available from Calbiochem-Novabiochem (UK) Ltd, Nottingham NG7 2QJ, UK. Purification may be effected by any one, or a combination of, techniques such as size exclusion chromatography, ion-exchange chromatography and (principally) reverse-phase high performance liquid chromatography. Analysis of peptides may be carried out using thin layer chromatography, reverse-phase high performance liquid chromatography, amino-acid analysis after acid hydrolysis and by fast atom bombardment (FAB) mass spectrometric analysis.

A further aspect of the invention provides a method of identifying a compound that modulates the activity of a polypeptide as defined in the first aspect of the invention, the method comprising contacting a compound with the polypeptide or a suitable variant, fragment, derivative or fusion thereof or a fusion of a variant, fragment or derivative thereof and determining whether the protein kinase activity of the said polypeptide is changed compared to the activity of the said polypeptide or said variant, fragment, derivative or fusion thereof or a fusion of a variant, fragment or derivative thereof in the absence of said compound.

It will be understood that it will be desirable to identify compounds that may modulate the activity of the polypeptide *in vivo*. Thus it will be understood that reagents and conditions used in the method may be chosen such that the interactions between the said polypeptide and its substrate are substantially the same as between human SKK4 and its substrate *in vivo*. An example of a substrate of said polypeptide is SAPK1.

In one embodiment, the compound decreases the activity of said polypeptide. For example, the compound may bind substantially reversibly or substantially irreversibly to the active site of said polypeptide. In a further example, the compound may bind to a portion of said polypeptide that is not the active site so as to interfere with the binding of the said polypeptide to its substrate. In a still further example, the compound may bind to a portion of said polypeptide so as to decrease said polypeptide's activity by an allosteric effect. This allosteric effect may be an allosteric effect that is involved in the natural regulation of the said polypeptide's activity.

In a further embodiment, the compound increases the activity of said polypeptide. For example, the compound may bind to a portion of said polypeptide that is not the active site so as to aid the binding of the said polypeptide to its substrate. In a still further example, the compound may bind to a portion of said polypeptide so as to increase said polypeptide's activity by an allosteric effect. This allosteric effect may be an allosteric effect that is involved in the natural regulation of the said polypeptide's activity.

Conveniently, the method makes use of the fact that SKK4 phosphorylates SAPK1 as described in Example 1 or Example 2, but any suitable substrate may be used.

Conveniently, the method makes use of an assay which may be substantially the same as that described in Example 1. In Example 1, phosphorylation of ATF2 by SAPK1 is measured. Alternatively but still

preferably, phosphorylation of GST-c-Jun(1-194) by SAPK1 may be measured. It is preferred that the SKK4 is recombinant SKK4.

A still further aspect of the invention provides a method of identifying a compound which binds to SAPK1 (or other substrate of the polypeptide as defined in the first aspect of the invention) and enhances or prevents its activation by the polypeptide as defined in the first aspect of the invention, the method comprising determining whether a compound enhances or prevents the interaction of SAPK1 (or other substrate of the polypeptide as defined in the first aspect of the invention) or a suitable fragment, variant, derivative or fusion thereof or a suitable fusion of a fragment, variant or derivative with the polypeptide as defined in the first aspect of the invention or determining whether the compound substantially blocks activation of SAPK1 (or other substrate of the polypeptide as defined in the first aspect of the invention) or a suitable fragment, variant, derivative or fusion thereof or a suitable fusion of a fragment, variant or derivative by the polypeptide as defined in the first aspect of the invention.

A still further aspect of the invention provides a method of identifying a compound which modulates the activation of the polypeptide as defined in the first aspect of the invention by an "upstream activator", for example MEKK. By "upstream activator" is meant a molecule that interacts with the polypeptide of the invention with the result that the protein kinase activity of the polypeptide of the invention is increased. It may be a polypeptide. Preferably, it is a physiological activator of native SKK4.

The method comprises determining whether a compound enhances or disrupts the interaction between (a) a polypeptide as defined in the first aspect of the invention or a suitable fragment, variant, derivative or fusion thereof or a suitable fusion of a fragment, variant or derivative and (b) an "upstream activator", for example MEKK, or a suitable variant, derivative, fragment or fusion thereof or a suitable fusion of a variant, derivative or fragment, or determining whether the compound substantially blocks activation of the said polypeptide or a suitable variant, fragment, derivative or fusion thereof, or a fusion of a said fragment, derivative or fusion by an "upstream activator" or a suitable variant, derivative, fragment or fusion thereof.

MEKK is a known activator of SKK1 that is shown here to also be an activator of the polypeptide of the invention, known as SKK4. Prior to the present invention, it was not known how SKK4 could be activated. By "activation of SKK4" it is meant that the ability of SKK4 to phosphorylate SAPK1 is increased following the treatment of SKK4, for example by MgATP and MEKK.

The sequence of MEKK is given in Lange-Carter *et al* (1993) *Science* **260**, 315-319, and its expression as a histidine-tagged fusion protein and purification using nickel-nitrilotriacetate-agarose is described in Meier *et al* (1996) and Example 1.

Thus a further aspect of the invention is the use of MEKK for the activation of the polypeptide of the invention.

A still further aspect of the invention provides a method of identifying a polypeptide that interacts with the protein kinase (polypeptide) of the invention, the method comprising 1) contacting a) the said protein kinase as defined in the first aspect of the invention or a suitable variant fragment, derivative or fusion thereof or a fusion of a variant, fragment or derivative thereof with b) a composition that may contain a polypeptide that interacts with the said protein kinase, 2) detecting the presence of a complex containing the said protein kinase and a polypeptide, and optionally 3) identifying any polypeptide bound to the said protein kinase.

In one embodiment, the composition may comprise material from cells. In particular, the cells may be selected from the following types: 1) cells which do not have SKK4 activity even when stimulated, 2) cells which have SKK4 activity after exposure to a stimulus, but which have not been so exposed and 3) cells of type 2 after exposure to the stimulus. Polypeptides that are found in a subset only of types 1-3 are of particular interest and may be characterised further. Such a peptide may be an activator of SKK4. Alternatively, it may be an inactivator of SKK4.

It will be appreciated that the method may be performed within a cell, for example using the yeast two hybrid system as is well known in the art. In this example, cDNAs copied from mRNA from the three cell types described above would be used.

A still further aspect of the invention provides a method of identifying a compound which blocks the activation of the polypeptide as defined in the first aspect of the invention by an interacting polypeptide, for example MEKK, the method comprising determining whether a compound

enhances or disrupts the interaction between (a) a protein kinase as defined in the first aspect of the invention or a suitable fragment, variant, derivative or fusion thereof or a suitable fusion of a fragment, variant or derivative and (b) said interacting polypeptide or a suitable variant, derivative, fragment or fusion thereof or a suitable fusion of a variant, derivative or fragment, or determining whether the compound substantially blocks activation of the polypeptide according to the first aspect of the invention or a suitable variant, fragment, derivative or fusion thereof, or a fusion of a said fragment, derivative or fusion by said interacting polypeptide or a suitable variant, derivative, fragment or fusion thereof.

Conveniently, the said polypeptide according to the first aspect of the invention or fragment, derivative, variant or fusion thereof used in the method is one which is produced by recombinant DNA technology. Similarly, it is preferred if the SAPK1 or fragment, derivative, variant or fusion thereof used in the method of identifying compounds that modulate activity of the said protein kinase is one which is produced by recombinant DNA technology. Similarly, it is preferred if MEKK or other "upstream activator" or fragment, derivative, variant or fusion thereof used in the method is one which is produced by recombinant DNA technology.

It will be appreciated that it may be necessary to activate the polypeptide of the invention prior to its use in assays. In a preferred embodiment the polypeptide of the invention (SKK4) is activated *in vitro* by treating the polypeptide with MEKK and MgATP, as described in Example 1. It is particularly preferred if the SKK4 is the recombinant polypeptide produced according to the methods of the invention.

It will be appreciated that by "suitable" we mean that the said components in the method are those that have interactions or activities which are substantially the same as those of SKK4 or SAPK1 or other substrates, or the upstream activator such as MEKK as the case may be but which may be more convenient to use in an assay. For example, fusions of SKK4 or SAPK1 are particularly useful since said fusion may contain a moiety which may allow the fusion to be purified readily.

It will be appreciated that the methods described may be performed in cells. "Reporter gene" constructs may be prepared by methods known to those skilled in the art, using the teaching herein. For example, a reporter gene construct may be made with a c-jun-dependent promoter sequence. This construct may be introduced together with an SKK4 construct into a cell line, in the parent cell line of which SAPK1 is activated in response to known stimuli, and in which the endogenous SKK4 gene has been inactivated. Alternatively the reporter gene construct could be introduced into the cell line in which SAPK1 is activated in response to known stimuli. The expression of the reporter gene will be dependent on the activity of SKK4 and thus the effect of compounds can be measured. In a further example, the reporter gene may be fatal to the cells, or alternatively may allow cells to survive under otherwise fatal conditions. Cell survival can then be measured, for example using colorimetric assays for mitochondrial activity, such as reduction of WST-1 (Boehringer). WST-1 is a formosan dye that undergoes a change in absorbance on receiving electrons via succinate dehydrogenase. In a further embodiment the yeast two-hybrid system is used.

The enhancement or disruption of the interaction between the said polypeptide of the invention and SAPK1 or an interacting polypeptide as defined above, or suitable derivatives, fragments, fusions or variants can be measured *in vitro* using methods well known in the art of biochemistry and include any methods which can be used to assess protein-protein interactions.

The said interaction can also be measured within a cell, for example using the yeast two hybrid system as is well known in the art.

It will be appreciated that the invention provides screening assays for drugs which may be useful in modulating the activity of SKK4 or its interactions with upstream activators. The compounds identified in the methods may themselves be useful as a drug or they may represent lead compounds for the design and synthesis of more efficacious compounds.

It will be appreciated that screening assays which are capable of high throughput operation will be particularly preferred. Examples may include the cell based assays described and protein-protein binding assays. A further example is an SPA-based (Scintillation Proximity Assay) system as described in Example 2.

A further aspect of the invention provides a compound identifiable by the screening methods of the invention. A still further aspect provides such a compound for use in medicine.

Before the present invention it was not appreciated how diverse stimuli may lead to activation of SKK4. It was therefore not known that the

SKK4 signalling pathway could potentially be modulated in such a way that responses to particular stimuli could be selectively affected. The motivation to attempt to identify compounds that modulate the signalling pathway was therefore lacking, as it would have appeared likely that such compounds could interfere with beneficial roles of the SKK4 signalling pathway, in addition to potentially detrimental ones.

We show here that SKK4 becomes activated in response to proinflammatory cytokines (interleukin-1, tumour necrosis factor) in human epithelial cells, whereas the other known activator of SAPK1 (SKK1/MKK4) is not. Since overproduction and/or inappropriate production of interleukin-1 and tumour necrosis factor is known to be a major cause of inflammatory diseases, such as rheumatoid arthritis, drugs that inhibit SKK4 are likely to suppress a number of the intracellular effects of these cytokines and may therefore be useful for treating inflammation.

A further aspect of the invention is the use of any of the screening methods of the invention in the identification of a molecule that may be useful in treating inflammatory disease.

It is believed that a compound identifiable by any of the screening methods of the invention may be useful in treating inflammatory disease. Inflammatory diseases include rheumatoid arthritis, psoriasis, septic shock, asthma and inflammatory bowel disease.

Thus, a further aspect of the invention provides a method of treating a patient with an inflammatory disease the method comprising administering

to the patient an effective amount of a compound identifiable by the screening methods of the invention.

A still further invention provides a use of a compound identifiable by the screening methods of the invention in the manufacture of a medicament for treating an inflammatory disease in a patient.

Thus, a further aspect of the invention provides a method of treating a patient with an inflammatory disease the method comprising administering to the patient an effective amount of a compound identifiable by the screening methods of the invention.

It is further believed that such compounds are useful in treating disease in which apoptosis is involved. For example, such compounds may suppress apoptosis, which may aid cell survival during or following cell damaging processes. Examples of such diseases include, but are not limited to, ischaemic disease, for example stroke and myocardial infarction, neural injury and myocardial infarction.

Some such compounds may aid apoptosis. Conditions in which aiding apoptosis may be of benefit include resolution of inflammation.

Thus, a further aspect of the invention provides a method of treating a patient with an ischaemic disease the method comprising administering to the patient an effective amount of a compound identifiable by the screening methods of the invention.

A still further invention provides a use of a compound identifiable by the screening methods of the invention in the manufacture of a medicament for treating an ischaemic disease in a patient.

Thus, a further aspect of the invention provides a method of treating a patient with an ischaemic disease the method comprising administering to the patient an effective amount of a compound identifiable by the screening methods of the invention.

The aforementioned compounds of the invention or a formulation thereof may be administered by any conventional method including oral and parenteral (e.g. subcutaneous or intramuscular) injection. The treatment may consist of a single dose or a plurality of doses over a period of time.

Whilst it is possible for a compound of the invention to be administered alone, it is preferable to present it as a pharmaceutical formulation, together with one or more acceptable carriers. The carrier(s) must be "acceptable" in the sense of being compatible with the compound of the invention and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free.

Thus, the invention also provides pharmaceutical compositions comprising the compound identifiable by the screening methods of the invention and a pharmaceutically acceptable carrier.

Further aspects of the invention provide a use of a polypeptide (protein kinase) as defined in the first aspect of the invention in a screening assay

for compounds which inhibit the activity of the said protein kinase or which block interactions of said protein kinase.

A further aspect of the invention provides a kit of parts that are useful in carrying out the screening methods.

The present invention will now be described in more detail with reference to the following Figures and Examples in which:

Figure 1: the nucleotide and predicted amino acid sequence of human SAP kinase kinase-4 (SKK4). Nucleotides are numbered in the 5′ to 3′ direction and amino acids are shown in single-letter code above the nucleotide sequence. The in-frame termination codon is marked by an asterisk.

Figure 2: the sequence comparison of human SKK4, human SKK1, human SKK2, human SKK3 and HEP from *D. melanogaster*. Amino acids were aligned and gaps were introduced to maximise the homology. Amino acid identities between at least three of the five sequences are indicated by boxed regions.

Figure 3: activation of SKK4 and SKK1/MKK4 in KB cells. (A) KB cells were incubated in the absence (control) or presence of the indicated agonist as described in Section 2.7 of Example 1 and SKK4 (open bars) and SKK1/MKK4 (filled bars) immunoprecipitated from the lysates and assayed for their ability to activate SAPK1/JNK1. The results are shown as means ± SEM for six dishes of ells with each agonist (two separate experiments). The basal activity of SKK4 and SKK1/MKK4 in

unstimulated cells was 5.0 ± 0.4 mU/mg and 2.0 ± 0.2 mU/mg, respectively. Abbreviations: - IL-1, interleukin-1; TNFα, tumour necrosis factor α; EGF, epidermal growth factor; PMA, phorbol myristate acetate. (B) SKK4 and SKK1/MKK4 were immunoprecipitated from extracts prepared from UV-irradiated KB cells. Where indicated, the antibodies were incubated with the peptides used to raise the anti-SKK4 or anti-SKK1/MKK4 antibodies. The results are presented as means ± SEM for at least three experiments.

Figure 4: GST-SKK4 is activated by MEKK and activates SAPK1/JNK, but not SAPK2a/p38. GST-SKK4 (0.25 μM) and GST-SKK1/MKK4 (0.1μM) were incubated with MgATP in the presence or absence of MEKK and then assayed for their ability to activate SAPK1/JNK (A) or SAPK2a/p38 (B) (Section 2.8 of Example 1). The activation of SAPK1/JNK and SAPK2a/p38 were monitored by the phosphorylation of GST-ATF2[19-96].

Figure 5: SKK4 immunoprecipitated from extracts prepared from UV-irradiated KB cells activates SAPK1/JNK, but not SAPK2/p38. SKK4 and SKK1/MKK4 immunoprecipitates were incubated with MgATP and SAPK2a/p38 or SAPK1/JNK. At the end of the reaction SAPK2a/p38 and SAPK1/JNK activity was then measured using ATF2 as substrate. The results are expressed as means ± SEM for three experiments.

Example 1: Purification, characterisation, cloning and expression of SKK4, and generation of antibodies binding to SKK4

Summary. A cDNA was cloned and expressed that encodes human stress-activated protein kinase kinase-4 (SKK4), a novel MAP kinase

kinase family member whose mRNA is widely expressed in human tissues. SKK4 activated SAPK1/JNK *in vitro*, but not SAPK2a/p38, SAPK2b/p38β, SAPK3/ERK6 or SAPK4. It appears to be the mammalian homologue of HEP, an activator of SAPK1/JNK in Drosophila. In human epithelial KB cells SKK4 and SKK1/MKK4 (another activator of SAPK1/JNK) were both activated by stressful stimuli, but only SKK4 was activated strongly by proinflammatory cytokines. The identification of SKK4 explains why the major SAPK1/JNK activator detected in many mammalian cell extracts is chromatographically separable from SKK1/MKK4.

Key words. SAPK, JNK, p38, MAP kinase, cytokine, stress

1. Introduction. Seven mitogen-activated protein kinase (MAPK) family members have been identified that are activated strongly by adverse stimuli (eg chemical, heat and osmotic shock, ultraviolet radiation and the protein synthesis inhibitor anisomycin) or by signals produced/released during infection (eg lipopolysaccharide and the proinflammatory cytokines interleukin-1 (IL-1) and tumour necrosis factor (TNF)), but only weakly (in most cell contexts) by polypeptide growth factors or phorbol esters (reviewed in [1]). For these reasons, they are termed stress-activated protein kinases (SAPKs).

The three isoforms of SAPK1/JNK bind tightly to the activation domain of c-Jun and are the only SAPKs that phosphorylate (at significant rates) the residues in this transcription factor that become phosphorylated *in vivo* in response to adverse stimuli and proinflammatory cytokines [204]. The two isoforms of SAPK2/p38 (SAPK2a/p38 and SAPK2b/p38β) are

inhibited specifically by the pyridinyl imidazoles SB 203580 and SB 202190 at submicromolar concentrations [4-6] and their physiological substrates include the transcription factors Elk1 [7], SAP1 [7], CHOP [8] and MEF2C [9], as well as MAPK-activated protein kinase-2 (MAPKAP-K2) [10], MAPKAP]K3 [11,12], MAPK-interacting protein kinase-1 (Mnk1) and Mnk2 [13,14]. Intracellular targets for MAPKAP-K2/MAPKAP-K3 include heat shock protein 27 [5,15], tyrosine hydroxylase [16] and the transcription factor CREB [17], while one of the substrates for Mnk1/Mnk2 is eukaryotic initiation factor eIF4E [13]. SAPK3/ERK6 [18,20] and SAPK4 [4,6] have only been identified recently and their physiological substrates are unknown. SAPK3 and SAPK4 are 60% identical to each other or to SAPK2a and SAPK2b, but they are not inhibited by SB 203580 and SB 202190 [4,6,18].

The SAPKs are activated by MAPK kinase (MKK) family members, termed here SAPK kinases (SKKs). The major activator of SAPK2a/p38, SAPK2b/p38β, SAPK3 and SAPK4 that has been detected biochemically is SKK3/MKK6 [4,18,21]23], although SAPK2/p38 can also be activated by SKK2/MKK3 *in vitro* [24]. Neither SKK2/MKK3 nor SKK3/MKK6 are capable of activating SAPK1/JNK, which is activated by a distinct enzyme termed SKK1/MKK4 [24,25]. SKK1/MKK4 is the only activator of SAPK1/JNK that can be detected biochemically in extracts prepared from PC12 cells that have been exposed to adverse stimuli [21], and in 293 cells the activation of transfected SAPK1/JNK by anisomycin can be prevented by overexpressing a catalytically inactive form of SKK1/MKK4 [25]. In addition, heat shock or anisomycin failed to activate SAPK1/JNK in murine embryonic stem cells lacking SKK1/MKK4 [26,27].

SKK1/MKK4 can activate SAPK2/p38 *in vitro* [24,28] but, in contrast to SAPK1/JNK which is activated strongly by cotransfection with MEK kinase (MEKK) [29] (an upstream activator of SKK1/MKK4), SAPK2/p38 is not activated [24] or activated much more weakly [30]. Moreover the activation of SAPK2/p38 by osmotic shock and anisomycin is unimpaired in murine embryonic stem cells lacking SKK1/MKK4 [26,27]. For these reasons, SKK1/MKK4 does not seem to be rate-limiting for the activation of SAPK2/p38 *in vivo*.

Although SKK1/MKK4 is clearly essential for the activation of SAPK1/JNK in some cells, the dominant activator of SAPK1/JNK detected biochemically in human epithelial KB cells [21] or in Rat 3Y1 fibroblasts [31] is a chromatographically distinct enzyme(s). Moreover, the activation of SAPK1/JNK by UV radiation and osmotic shock in murine embryonic stem cells lacking SKK1/MKK4 was either unimpaired [26] or only inhibited partially [27]. These observations indicate that mammalian cells contain at least one further activator of SAPK1/JNK distinct from SKK1/MKK4. In this Example we have cloned a novel MKK family member, termed here SKK4, that is activated by stressful stimuli and proinflammatory cytokines and which activates SAPK1/JNK, but not other SAPKs.

2. Materials and Methods

2.1 Materials. An *E. coli* plasmid encoding a fusion protein comprising glutathione S-transferase (GST) linked to residues 19-96 of activating transcription factor-2 (GST-ATF2[19-96]) was provided by Dr N Jones (ICRF, London) and a plasmid encoding GST-SKK1/MKK4 by

Dr J Woodgett (Ontario Cancer Institute, Toronto). A plasmid encoding the maltose-binding protein (MalE) linked to the Xenopus homologue of SAPK2a/p38 (MalE-Mpk2) was a gift from Dr A R Nebreda (EMBL, Heidelberg, Germany), while a plasmid encoding MEKK preceded by six histidine residues (6-His-MEKK) was provided by Dr G Johnson (National Jewish Centre for Immunology and Respiratory Medicine, Denver, USA). All plasmids were transformed into *E. coli* strain BL21 (DE3), and expressed and purified as described [4,18,21]. 6-His SAPK1/JNK1γ [32] was expressed and purified as reported previously [33]. PKI, the specific peptide inhibitor of cAMP-dependent protein kinase (TTYADFIASHGRTGRRNAIHD) was synthesised by Mr F B Caudwell in the MRC Protein Phosphorylation Unit in Dundee and other peptides by Dr Graham Bloomberg, University of Bristol, UK.

2.2 cDNA cloning and sequencing. Two oligonucleotides corresponding to part of the EST encoded by GenBank ID H85962, (forward, 5'-GACGGATCCGACCCCACCAAGCCGGACTTT; reverse, 5'-GGCCAAGCTTGTCTTTGACGAAGGACTGGAA) were used to amplify a 209 base pair fragment from a λgt10 human skeletal muscle cDNA library (Clontech). The nucleotide sequence of this product was identical to that in the database. The PCR fragment was ^{32}P-labelled by random priming and used to probe the same cDNA library at high stringency. One strongly hybridising plaque was identified after screening 500,000 plaques. This clone was isolated, the phage DNA purified, and the insert cloned into pBluescript (Stratagene) and sequenced using an Applied Biosystems model 373A DNA sequencer.

2.3 Expression of GST-SKK4 in *E. coli*. The open reading frame of SKK4 was amplified by PCR and subcloned as a BamHI/EcoRI fragment into the expression vector pGEX4T-3 (Pharmacia) followed by transformation into *E. coli* strain BL21 (DE3). Expression and purification of GST-SKK4 were carried out as described in [34]. The purified protein was dialysed against 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.03% (m/v) Brij35, 0.1% (v/v) 2-mercaptoethanol and 50% (v/v) glycerol, and stored unfrozen at -20 °C at 2 mg/ml.

2.4 Preparation of anti-SKK4 and anti-SKK1/MKK4 antibodies. Peptides were synthesised corresponding to the C-terminal 12 residues of human SKK4 (GVLSQPHLPFFR) and the last 14 residues of SKK1/MKK4 (DQMPATPSSPMYVD) [24], coupled to both bovine serum albumin and keyhole limpet haemocyanin using glutaraldehyde and injected into sheep at the Scottish Antibody Production Unit (Carluke, Ayrshire, UK). The anti-SKK4 and anti-SKK1/MKK4 antibodies were purified on peptide antigen-CH-Sepharose columns [22].

2.5 Cell culture and cell lysis. KB cells were cultured [18] and exposed for 15 min to osmotic stress (0.5 M sorbitol) or proinflammatory cytokines (20 ng/ml IL-1α or 100 ng/ml TNFα), or for 30 min to anisomycin (10 μg/ml), or for 10 min to 100 ng/ml epidermal growth factor (EGF) or 300 ng/ml phorbol myristate acetate (PMA). UV-C irradiation was carried out at 60 J/m2 and the cells then incubated for a further 30 min at 37 °C. Cells were lysed as described [21].

2.6 Immunoprecipitation of SKK4 and SKK1/MKK4. KB cell lysates (250 :g) were incubated for 60 min at 4 °C with 10 μg of affinity

purified antibody coupled to 5 μl protein G-Sepharose. The protein G-Sepharose immunoprecipitates were washed twice with 1 ml lysis buffer containing 0.5 M NaCl, twice with lysis buffer without NaCl and assayed as described below. In control experiments, antibodies bound to protein G-Sepharose were incubated for 30 min at 4 °C with the peptide immunogen (1 mg/ml) prior to the addition of cell lysate.

2.7 Assay of SKK1/MKK4 and SKK4 immunoprecipitates. Immunoprecipitates (~6.5 μl) were incubated on a shaking platform for 30 min at 30 °C with 1 :l of 20 μM 6-His-SAPK1/JNK in 50 mM Tris/HCl (pH 7.4), 0.1 mM EGTA, 0.03% (m/v) Brij-35, 0.1% (v/v) 2-mercaptoethanol and 5% (v/v) glycerol, and the reactions initiated with 2.5 μl of 40 mM magnesium acetate-0.4 mM unlabelled ATP. After 30 min, the active SAPK1/JNK generated was assayed by adding 40 μl of a solution containing 31.25 mM Tris/HCl (pH 7.4), 0.125 mM EGTA, 1.25 mM sodium orthovanadate, 3.1 μM PKI, 0.1% (by vol) 2-mercaptoethanol and 0.25 mg/ml GST-ATF2[19-96], 12.5 mM magnesium acetate and 0.125 mM [(γ^{32}P]ATP. After 30 min at 30 °C reactions were stopped by adding 5 :l of 6% (m/v) SDS, 400 mM Tris/HCl (pH 6.8), 50% (v/v) glycerol, 5% (v/v) 2-mercaptoethanol and 0.2% (m/v) bromophenol blue. A 40 μl aliquot was withdrawn, electrophoresed on a 10% SDS/polyacrylamide gel and autoradiographed. The ^{32}P-labelled band corresponding to GST-ATF[19-96] was excised and counted. One unit of SKK1/MKK4 or SKK4 activity was that amount which increased the activity of SAPK1/JNK by 1 U/min. One Unit of SAPK1/JNK was that amount which incorporated 1 nmol of phosphate into GST-ATF2[19-96] in one min.

SKK1/MKK4 and SKK4 were also assayed for their ability to activate Xenopus SAPK2a. The assay was identical except that SAPK2a (2 μM) replaced SAPK1/JNK.

2.8 Activation and assay of expressed GST-SKK4 and GST-SKK1/MKK4. Each GST fusion protein (4 μM) was activated by incubation for 60 min at 30 °C with MEKK (1 μM) in 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 10 mM magnesium acetate and 0.1 mM unlabelled ATP. Activated GST-SKK4 and GST-SKK1/MKK4 were then measured by their ability to activate SAPK1/JNK or SAPK2a/p38, which were assayed by the phosphorylation of ATF2. The assays (50 μl) were carried out at 30 °C and comprised 0.1 μM GST-SKK4 or GST-SKK1/MKK4, 0.2 μM 6-His SAPK1/JNK, 0.2 mg/ml GST-ATF2[19-96], 50 mM Tris/HCl pH 7.5, 0.1 mM EGTA, 0.1% (v/v) 2-mercaptoethanol, 10 mM magnesium acetate and 0.1 mM [γ^{32}P]ATP. The reactions were terminated by spotting 40 μl on to 2 x 2 cm squares of phosphocellulose p81 paper followed by immersion into 75 mM phosphoric acid. After washing the papers several times in phosphoric acid followed by immersion in acetone, the papers were dried and counted.

3. Results

3.1 Molecular cloning of SKK4. To identify novel members of the SKK family, we used the DNA sequence encoding SKK1/MKK4 to interrogate a number of EST databases. This search identified a 250 bp sequence (GenBank ID H85962) that was distinct from any previously identified SKK homologue, but was much more closely related to

members of this family than to any other protein kinase. A suitable PCR probe (see Methods) was therefore used to screen a human skeletal muscle cDNA library. This resulted in the isolation of a 1.2 kb clone that encoded a novel MKK family member, hereafter termed SKK4. The nucleotide and deduced amino acid sequence of human SKK4 is shown in Fig 1. The open reading frame encodes a protein of 333 residues, with a predicted molecular mass of 37.9 kDa. It possesses all the conserved amino acid domains (I-XI) characteristic of protein kinases and shows 62% identity with the Drosophila MKK homologue HEP (see Discussion), 48% identity with human SKK1/MKK4, 39% identity with human SKK2/MKK3, and 41% identity with human SKK3/MKK6 (Fig 2). The sequence identity with MKK1 and MKK2 is 36% and 35%, respectively. Ser185 and Thr189 in kinase subdomain VIII are in an equivalent position to the Ser/Thr residues in other MKK family members, whose phosphorylation is required to generate enzymatic activity.

Hybridisation of ^{32}P-labelled SKK4 cDNA to multiple tissue Northern blots (Clontech) showed an RNA transcript of ~4 kb that was present in heart, brain, placenta, lung, liver, skeletal muscle, kidney and pancreas (data not shown). The presence of ESTs in databases revealed that SKK4 is also expressed in retina and germinal centre B-cells. Thus SKK4 and mRNA is widely expressed in mammalian cells.

3.2 SKK4 is activated by proinflammatory cytokines and stressful stimuli. An antibody was raised against the unique C-terminal sequence of SKK4 (Section 2.4) and used to immunoprecipitate SKK4 from KB cell extracts after stimulation with a variety of agonists (Fig 3). These studies revealed that SKK4 was activated rapidly in KB cells in response to the

same stimuli that trigger the activation SAPK1/JNK [21], namely proinflammatory cytokines and stressful stimuli (UV radiation, osmotic shock and anisomycin). SKK4 was activated weakly by EGF, but not by PMA. SKK4 was also activated by stressful stimuli in COS cells (data not shown). SKK4 was activated to a greater extent by interleukin-1 (IL-1) and tumour necrosis factor (TNFα) than by UV irradiation, osmotic shock or anisomycin.

SKK1/MKK4, another activator of SAPK1/JNK (see Introduction) is also present in KB cells and, like SKK4, was activated by UV irradiation, osmotic shock and anisomycin. However, in contrast to SKK4, SKK1/MKK4 was hardly activated by interleukin-1, TNFα or EGF (Fig 3A).

The specificities of the antibodies used in these experiments were established by the finding that immunoprecipitation of SKK4 was prevented by incubating the anti-SKK4 antibody with the SKK4 peptide immunogen used to generate it, but not with the SKK1/MKK4 peptide immunogen. Conversely, immunoprecipitation of SKK1/MKK4 was prevented by incubating the anti-SKK1/MKK4 antibody with the SKK1/MKK4 peptide immunogen, but not the SKK4 peptide immunogen (Fig 3B).

3.3 Activation and substrate specificity of SKK4. GST-SKK4 was expressed in *E. coli* (Section 2.3) and 10 mg of purified enzyme could be isolated from 500 ml of bacterial culture. The preparation showed a single protein-staining band with the predicted molecular mass of 64 kDa (data not shown). GST-SKK4 was inactive, but could be activated by

incubation with MgATP and MEKK (Fig 4), an enzyme that also activates SKK1/MKK4 [29]. The activated GST-SKK4 was able to activate SAPK1/JNK *in vitro*, but not SAPK2a/p38 (Fig 4), SAPK2b/p38β, SAPK3/ERK6 or SAPK4 (data not shown). In contrast, activated GST-SKK1/MKK4 activated SAPK2a/p38 as well as SAPK1/JNK (Fig 4).

SKK4 and SKK1/MKK4 immunoprecipitated from the lysates of UV-stimulated KB cells had the same specificity as the bacterially expressed enzymes. Thus SKK4 activated SAPK1/JNK but not SAPK2a/p38, while SKK1/MKK4 activated both enzymes (Fig 5).

4. Discussion

In this Example we have cloned a novel MKK homologue that is activated strongly by proinflammatory cytokines and stressful stimuli, and has therefore been termed SKK4. SKK4 expressed in *E. coli* and activated *in vitro*, or immunoprecipitated from KB cell extracts activated SAPK1/JNK, but was unable to activate other SAPKs, suggesting that SAPK1/JNK may be a physiological substrate of SKK4. This view is reinforced by the striking similarity between SKK4 and the Drosophila MKK homologue, termed HEP (Fig 2). HEP was identified by genetic dissection of a signalling pathway that is required for dorsal closure during early embryonic development and shown to be situated "upstream" of Drosophila JNK [35-37]. Thus HEP is almost certainly the enzyme responsible for activating JNK in Drosophila. The generation of mice lacking SKK4 will be needed to find out if SKK4 also plays an essential role in mammalian embryonic development.

SKK4 is activated *in vitro* by MEKK (Fig 4) one of many protein kinases [38] that have been shown to activate SKK1/MKK4 *in vitro* and to trigger the activation of SAPK1/JNK in cotransfection experiments. These observations, and the find that overexpression of inactivate mutants of SKK1/MKK4 prevent the activation of SAPK1/JNK by MEKK, other upstream activities and stressful stimuli [25,29,38], suggested that SKK1/MKK4 mediates the activation of SAPK1/JNK *in vivo*. However, since the inactive mutant of SKK1/MKK4 probably exerts its dominant negative effect by binding to SAPK1/JNK, it remained possible that MEKK and other upstream activators trigger the activation of SAPK1/JNK via another MKK homologue. This view was confirmed by biochemical experiments which show that the major activator(s) of SAPK1/JNK in several cell extracts is chromatographically separable from SKK1/MKK4 [21,31], and by the finding that UV radiation and osmotic shock can still trigger the activation of SAPK1/JNK in stem cells from mice that do not express SKK1/MKK4 [26,27]. The present work indicates that one of the additional SAPK1/JNK activators is likely to be SKK4. Whether mammalian cells express additional MKK homologues that activate SAPK1/JNK remains to be established.

The present work (Fig 3) shows that SKK4 is activated strongly by proinflammatory cytokines as well as stressful stimuli in KB cells, whereas SKK1/MKK4 is activated by stress stimuli only. These observations suggest that SKK4 may be a more attractive target for an anti-inflammatory drug than SAPK1/JNK because inhibitors of SKK4 may prevent the activation of SAPK1/JNK by proinflammatory cytokines, without affecting the activation of SAPK1/JNK by other agents.

References

1. Cohen, P. (1997) *Trends Cell Biol.* (in press).
2. Kyriakis, J.M. and Avruch, J. (1996) *Bioessays* **18**, 567-577.
3. Karin, M. (1995) *J. Biol. Chem.* **270**, 16483-16486.
4. Goedert, M. *et al* (1997) *EMBO J.* **16**, 3563-3571.
5. Cuenda, A. *et al* (1995) *FEBS Lett.* **364**, 229-233.
6. Kumar, S. *et al* (1997) *Biochem. Biophys. Res. Comm.* **235**, 533-538.
7. Price, M.A. *et al* (1996) *EMBO J.* **15**, 6652-6563.
8. Wang, X.Z. and Ron, D. (1996) *Science* **272**, 1347-1349.
9. Han, J. *et al* (1997) *Nature* **386**, 296-299.
10. Rouse, J. *et al* (1994) *Cell* **78**, 1027-1037.
11. McLaughlin, M.M. *et al* (1996) *J. Biol. Chem.* **271**, 8488-8492.
12. Clifton, A.D. *et al* (1996) *FEBS Lett.* **392**, 209-214.
13. Waskiewicz, A.J. *et al* (1997) *EMBO J.* **16**, 1909-1920.
14. Fukunaga, R. and Hunter, T. (1996) *EMBO J.* **16**, 1921-1933.
15. Guay, J. *et al* (1997) *J. Cell Sci.* **110**, 357-368.
16. Thomas, G. *et al* (1997) *Eur. J. Biochem.* (in press).
17. Tan, Y. *et al* (1996) *EMBO J.* **15**, 101-114.
18. Cuenda, A. *et al* (1997) *EMBO J.* **16**, 295-305.
19. Lechner, C. *et al* (1996) *Proc. Natl. Acad. Sci. USA* **93**, 4355-4359.
20. Mertens, S. *et al* (1996) *FEBS Lett.* **383**, 273-276.
21. Meier, R. *et al* (1996) *Eur. J. Biochem.* **236**, 796-805.
22. Cuenda, A. *et al* (1996) *EMBO J.* **15**, 4156-4164.
23. Moriguchi, T. *et al* (1996) *J. Biol. Chem.* **271**, 26981-26988.
24. Dérijard, B. *et al* (1995) *Science* **267**, 682-684.

25. Sanchez, I. *et al* (1994) *Nature* **372**, 794-798.

26. Nishina, H. *et al* (1997) *Nature* **385**, 350-353.

27. Yang, D. *et al* (1997) *Proc. Natl. Acad. Sci. USA* **94**, 3004-3009.

28. Doza, Y.N. *et al* (1995) *FEBS Lett.* **364**, 223-228.

29. Yan, M. *et al* (1994) *Nature* **372**, 798-800.

30. Xu, S. *et al* (1996) *Proc. Natl. Acad. Sci. USA* **93**, 5291-5295.

31. Moriguchi, T. *et al* (1995) *J. Biol. Chem.* **270**, 12969-12972.

32. Dérijard, B. *et al* (1994) *Cell* **76**, 1025-1037.

33. Goedert, M. *et al* (1997) *FEBS Lett.* **409**, 57-62.

34. Alessi, D.R. *et al* (1995) *Methods Enzymol.* **255**, 279-290.

35. Gilse, B. *et al* (1995) *Cell* **83**, 451-461.

36. Riesgo-Escovar, J.R. *et al* (1996) *Gene Dev.* **10**, 2759-2768.

37. Sluss, H.K. *et al* (1996) *Gene Dev.* **10**, 2745-2758.

38. Fanger, G.R. *et al* (1997) *Curr. Opin. Genetic. Dev.* 7, 67-74.

39. Raingeaud, J. *et al* (1996) *Mol. Cell. Biol.* **16**, 1247-1255.

Example 2: Alternative protein kinase assays

SKK4 is assayed routinely by phosphorylation of SAPK1, as described in Meier *et al.,* (1996). 10 μl of a solution containing SKK4 and inactive SAPK1 (equivalent to 100mU before inactivation with PTP 1B) in 20 mM sodium glycerol 2-phosphate pH 7.4, 1.0 mM EGTA, 0.03% (by mass) Brij-35, 5% (by vol.) glycerol, 1 mM benzamadine, 0.1% (by vol.) 2-mercaptoethaonol, 1 mM sodium orthovanadate, 1 μM microcystin-LR was incubated for 3 min at 30 °C, and the reaction initiated with 2 μl 60 mM $MgCl_2$, 0.6 mM unlabeled ATP. After 30 min at 30 °C, a 5 μl aliquot was withdrawn and assayed for SAPK1 activity as described

below. Control incubations were performed in which SKK4 or inactive SAPK1 were omitted. One unit (U) of SKK4 was that amount which increased SAPK1 activity by 1 U/min.

SAPK1 is assayed by phosphorylation of GST-c-Jun(1-194), as described in Meier *et al.*, (1996). A solution (40 μl) containing SAPK1 and 200 μg/ml GST-c-Jun(1-194) in 25 mM Tris/HCl, 20 mM sodium glycerol 2-phosphate pH 7.4, 0.1 mM EGTA, 1 mM sodium orthovanadate, 2.5 μM PKI, was incubated for 3 min at 30 °C and the assay initiated with 10 μl 50 mM $MgCl_2$, 0.5 mM [γ-^{32}P]ATP (5x10^5 cpm/nmol).

After a further 20 min, a 40 μl aliquot was withdrawn, spotted on to a square (1.5 x 1.5 cm) of Whatman P81 phosphocellulose paper and immersed immediately in 75 mM phosphoric acid. After washing the papers several times in 75 mM phosphoric acid followed by immersion in acetone, ^{32}P radioactivity incorporated into c-Jun(1-194) was measured. One unit (U) of SAPK1 activity was that amount which catalysed the phosphorylation of 1 nmol GST-c-Jun(1-194) in 1 min.

Alternatively, a Scintillation Proximity Assay (SPA) system (Amersham International) is used to assess the incorporation of ^{32}P radioactivity into cJun(1-194). In this system, the sample is mixed with beads comprising scintillant and antibodies that bind GST-c-Jun(1-194). Conveniently this is done in a 96-well format. The plate is then counted using a suitable scintillation counter, using known parameters for ^{32}P SPA assays. Only ^{32}P that is in proximity to the scintillant, i.e. only that bound to GST-c-Jun(1-194) that is then bound by the antibody, is detected.

Example 3: Assay for compounds which modulate SKK4 activity

An assay is set up with SAPK1, as described in Example 1 or Example 2. Compounds are tested in the assay and those that give rise to inhibition or activation of SKK1 are selected for further study. To confirm that the any effects observed are not due to effects on SAPK1, compounds are tested for effects on activated and inactive SAPK1.

Example 4: Assay for polypeptides that interact with SKK4

A yeast two hybrid assay system is set up to identify polynucleotides encoding polypeptides that are capable of associating with SKK4 in a stable enough manner to allow transcriptional activation to occur. The polynucleotides are (in separate experiments) cDNAs copied from mRNA from cells that are capable of expressing SKK4, before or after stimulation capable of activating SKK4, and from cells which do not express SKK4. Interactions which are found in a subset only of these cell types are of particular interest.

The polypeptide encoded by the polynucleotide is determined by sequencing the insert by the Sanger method as described in Example 1 to obtain a predicted amino acid sequence.

REFERENCES

Beyaert, R. *et al* (1996) "The p38/RK mitogen-activated protein kinase pathway regulates interleukin-6 synthesis in response to tumour necrosis factor" *EMBO J.* **15**, 1914-1923.

Clifton, A.D. *et al* (1996) "A comparison of the substrate specificity of MAPKAP kinase-2 and MAPKAP kinase-3 and their activation by cytokines and cellular stress" *FEBS Lett.* **392**, 209-214.

Cohen, P. (1997) "Functions of mitogen- and stress-activated protein kinase cascades in mammalian cells identified by the use of specific inhibitors" *Trends Cell Biol.*, in press.

Cuenda, A. *et al* (1995) "SB 203580 is a specific inhibitor of a MAP kinase homologue which is stimulated by cellular stresses and interleukin-1" *FEBS Lett.* **364**, 229-233.

Cuenda, A. *et al* (1996) "Purification and cDNA cloning of SAPKK3, the major activator of RK/p38 in stress- and cytokine- stimulated monocytes and epithelial cells" *EMBO J.* **15**, 4156-4164.

Cuenda, A. *et al* (1997) "Activation of stress-activated protein kinase-3 (SAPK3) by cytokines and cellular stresses is mediated via SAPKK3 (MKK6); comparison of the specificities of SAPK3 and SAPK2 (RK/p38)" *EMBO J.* **16**, 295-305.

Dérijard, B. *et al* (1994) "JNK1: a protein kinase stimulated by UV light and Ha-Ras that binds and phosphorylates the c-Jun activation domain" *Cell* **76**, 1025-1037.

Dérijard, B. *et al* (1995) "Independent human MAP kinase signal transduction pathways defined by MEK and MKK isoforms" *Science* **267**, 682-684.

Doza, Y.N. *et al* (1995) "Activation of the MAP kinase homologue RK requires the phosphorylation of Thr-180 and Tyr-182 and both residues are phosphorylated in chemically stressed KB cells" *FEBS Lett.* **364**, 223-228.

Freshney, N.W. *et al* (1994) "Interleukin-1 activates a novel protein kinase cascade that results in the phosphorylation of hsp27" *Cell* **78**, 1039-1049.

Fukunaga, R. and Hunter, T. (1997) "Mnkl, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates" *EMBO J.* **16**, in press.

Ginty, D.D. *et al* (1994) "Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB" *Cell* **77**, 713-725.

Goedert, M. *et al* (1997) "Assignment of the human stress-activated protein kinase-3 gene (*SAPK3*) to chromosome 22q13.3 by fluorescence *in situ* hybridization" *Genomics*, in press.

Gould, G.W. *et al* (1995) "The activation of distinct mitogen-activated protein kinase cascades is required for the stimulation of 2-deoxyglucose uptake by interleukin-1 and insulin-like growth factor-1 in KB cells" *Biochem. J.* **311**,735-738.

Gupta, S. *et al* (1995) "Transcription factor ATF2 regulation by the JNK signal transduction pathway" *Science* **267**, 389-393.

Han, J. *et al* (1994) "A MAP kinase targeted by endotoxin and hyperosmolarity in mammalian cells" *Science* **265**, 808-811.

Han, J. *et al* (1996) "Characterization of the structure and function of a novel MAP kinase kinase (MKK6)" *J. Biol. Chem.* **271**, 2886-2891.

Hazzalin, C.A. (1996) "Essential role for p38/RK in stress-induced nuclear responses" *Curr. Biol.* **6**, 1028-1031.

Hibi, M. *et al* (1993) "Identification of an oncoprotein- and UV-responsive protein kinase that binds and potentiates the c-Jun activation domain" *Genes Dev.* **7**, 2135-2148.

Huot, J. *et al* (1995) "Characterization of 45-kDa/54-kDa HSP27 kinase, a stress-sensitive kinase which may activate the phosphorylation-dependent protective function of mammalian 27-kDa heat shock protein HSP27" *Eur. J. Biochem.* **227**,416427.

Jiang, Y. *et al* (1996) "Characterization of the structure and function of a new mitogen-activated protein kinase (p38β)" *J. Biol. Chem.* **271**, 17920-17926.

Kyriakis, J.M. (1994) "The stress-activated protein kinase subfamily of c-Jun kinases" *Nature* **369**, 156-160.

Lechner, C. (1996) "ERK6, a mitogen-activated protein kinase involved in C2C12 myoblast differentiation" *Proc. Natl. Acad. Sci. USA* **93**,4355-4359.

Lee, J.C. *et al* (1994) "A protein kinase involved in the regulation of inflammatory cytokine biosynthesis" *Nature* **372**, 739-746.

Li, Z. *et al* (1996) "The primary structure of p38: a new member of p38 group of MAP kinases" *Biochem. Biophys. Res. Commun.* **228**, 334-340.

Ludwig *et al* (1996) "3pK, a novel mitogen-activated protein (MAP) kinase-activated protein kinase, is targeted by three MAP kinase pathways" *Mol. Cell Biol.* 6687-6697

McLaughlin, M.M. *et al* (1996) "Identification of mitogen-activated protein (MAP) kinase-activated protein kinase-3, a novel substrate of CSBP p38 MAP kinase" *J. Biol. Chem.* **271**, 8488-8492.

Meier, R. *et al* (1996) "Cellular stresses and cytokines activate multiple mitogen-activated protein kinase kinase homologues in PC12 and KB cells" *Eur. J. Biochem.* **236**, 796-805.

Mertens, S. *et al* (1996) "SAP kinase-3, a new member of the family of mammalian stress-activated protein kinases" *FEBS Lett.* **383**, 273-276.

Moriguchi, T. *et al* (1995) "Evidence for multiple activators for stress-activated protein kinases/c-Jun amino-terminal kinases" *J. Biol. Chem.* **270**, 12969-12972.

Moriguchi, T. *et al* (1996) "A novel kinase cascade mediated by mitogen-activated protein kinase kinase 6 and MKK3" *J. Biol. Chem.* **271**,13675-13679.

Price, M.A. *et al* (1996) "The p38 and ERK MAP kinase pathways cooperate to activate Ternary Complex Factors and c-fos transcription in response to UV light" *EMBO J.* **15**, 6552-6563.

Pulverer, B.J. *et al* (1991) "Phosphorylation of c-jun mediated by MAP kinases" *Nature* **353**, 670-674.

Raingeaud, J. *et al* (1996) "MKK3-and MKK6-regulated gene expression is mediated by the p38 mitogen-activated protein kinase signal transduction pathway" *Mol. Cell. Biol.* **16**, 1247-1255.

Rouse, J. *et al* (1994) "A novel kinase cascade triggered by stress and heat shock that stimulates MAPKAP kinase-2 and phosphorylation of the small heat shock proteins" *Cell* **78**, 1027-1037.

Saklatvala, J. *et al* (1996) "Role of p38 mitogen-activated protein kinase

in platelet aggregation caused by collagen or a thromboxane analogue" *J. Biol. Chem.* **271**, 6586-6589.

Sanchez, I. *et al* (1994) "Role of SAPK/ERK kinase-1 in the stress-activated pathway regulating transcription factor c-Jun" *Nature* **372**, 794-798.

Stein, B. *et al* (1996) "Cloning and characterisation of MEK6, a novel member of the mitogen-activated protein kinase kinase cascade" *J. Biol. Chem.* **271**, 11427-11433.

Stokoe, D. *et al* (1993) "The substrate specificity and structure of mitogen-activated protein (MAP) kinase-activated protein kinase-2" *Biochem J.* **269**, 842-849.

Tan, Y. *et al* (1996) "FGF and stress regulate CREB and ATF-1 via a pathway involving p38 MAP kinase and MAPKAP kinase-2" *EMBO J.* **15**,4629-4642.

Wang, X.Z. and Ron D. (1996) "Stress-induced phosphorylation and activation of the transcription factor CHOP (GADDl53) by p38 MAP kinase" *Science* **272**, 1347-1348.

Waskiewicz, A.J. *et al* (1997) "Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2" *EMBO J.* **16**, 1909-1920.

Yan, M. *et al* (1994) "Activation of stress-activated protein kinase by MEKKl phosphorylation of its activator SEK1" *Nature* **372**, 798-800.

Yashar, B.M. *et al* (1993) "Novel members of the mitogen-activated protein kinase activator family in *Xenopus laevis*" *Mol. Cell. Biol.* **13**, 5738-5748.

Zervos, A.S. *et al* (1995) "Mxi2, a mitogen-activated protein kinase that recognizes and phosphorylates Max protein" *Proc. Natl. Acad. Sci. USA* **92**, 10531-10534.

CLAIMS

1. A substantially pure polypeptide comprising the amino acid sequence

M E S I E I D Q K L Q E I M K Q T G Y L
T I G G Q R Y Q A E I N D L E N L G E M
G S G T C G Q V W K M R F R K T G H V I
A V K Q M R R S G N K E E N K R I L M D
L D V V L K S H D C P Y I V Q C F G T F
I T N T D V F I A M E L M G T C A E K L
K K R M Q G P I P E R I L G K M T V A I
V K A L Y Y L K E K H G V I H R D V K P
S N I L L D E R G Q I K L C D F G I S G
R L V D S K A K T R S A G C A A Y M A P
E R I D P P D P T K P D Y D I R A D V W
S L G I S L V E L A T G Q F P Y K N C K
T D F E V L T K V L Q E E P P L L P G H
M G F S G D F Q S F V K D C L T K D H R
K R P K Y N K L L E H S F I K R Y E T L
E V D V A S W F K D V M A K T E S P R T
S G V L S Q P H L P F F R,

or a variant, fragment, fusion or derivative thereof, or a fusion of a said variant or fragment or derivative.

2. A variant of the polypeptide according to Claim 1 wherein the amino acid sequence of said variant has at least 99.1% identity with the given amino acid sequence.

3. A variant of the polypeptide according to Claim 2 wherein the amino acid sequence of said variant has at least 99.4% identity with the given amino acid sequence.

4. A variant of the polypeptide according to Claim 3 wherein the amino acid sequence of said variant has at least 99.7% identity with the given amino acid sequence.

5. A recombinant polynucleotide encoding a polypeptide as defined in any one of Claims 1 to 4 or encoding a variant or fragment or derivative or fusion of said polypeptide or a fusion of a said variant or fragment or derivative.

6. A recombinant polynucleotide comprising the nucleotide sequence
ATGGAGAGCATTGAGATTGACCAGAAGCTGCAGGAGATCATGAAGCAGACGGGCTACC
TGACCATCGGGGGCCAGCGCTACCAGGCAGAAATCAACGACCTGGAGAACTTGGGCGA
GATGGGCAGCGGCACCTGCGGCCAGGTGTGGAAGATGCGCTTCCGGAAGACCGGCCAC
GTCATTGCCGTTAAGCAAATGCGGCGCTCCGGGAACAAGGAGGAGAACAAGCGCATCC
TCATGGACCTGGATGTGGTGCTGAAGAGCCACGACTGCCCCTACATCGTGCAGTGCTT
TGGGACGTTCATCACCAACACGGACGTCTTCATCGCCATGGAGCTCATGGGCACCTGC
GCTGAGAAGCTCAAGAAGCGGATGCAGGGCCCCATCCCCGAGCGCATTCTGGGCAAGA
TGACAGTGGCGATTGTGAAGGCGCTGTACTACCTGAAGGAGAAGCACGGTGTCATCCA
CCGCGACGTCAAGCCCTCCAACATCCTGCTGGACGAGCGGGGCCAGATCAAGCTCTGC
GACTTCGGCATCAGCGGCCGCCTGGTGGACTCCAAAGCCAAGACGCGGAGCGCCGGCT
GTGCCGCCTACATGGCACCCGAGCGCATTGACCCCCCAGACCCCACCAAGCCGGACTA
TGACATCCGGGCCGACGTATGGAGCCTGGGCATCTCGTTGGTGGAGCTGGCAACAGGA
CAGTTTCCCTACAAGAACTGCAAGACGGACTTTGAGGTCCTCACCAAAGTCCTACAGG
AAGAGCCCCCGCTTCTGCCCGGACACATGGGCTTCTCGGGGGACTTCCAGTCCTTCGT
CAAAGACTGCCTTACTAAAGATCACAGGAAGAGACCAAAGTATAATAAGCTACTTGAA
CACAGCTTCATCAAGCGCTACGAGACGCTGGAGGTGGACGTGGCGTCCTGGTTCAAGG
ATGTCATGGCGAAGACTGAGTCACCGCGGACTAGCGGCGTCCTGAGCCAGCCCCACCT
GCCCTTCTTCAGG,
or a variant, fragment, fusion or derivative thereof.

7. A polynucleotide according to Claim 5 or 6 which contains no introns.

8. A replicable vector comprising a polynucleotide as defined in any one of Claims 5 to 7.

9. A host cell comprising a recombinant polynucleotide or a replicable vector as defined in any one of Claims 5 to 8.

10. A method of making a polypeptide, or a variant, fragment, derivative or fusion thereof or fusion of a said variant or fragment or derivative the method comprising culturing a host cell as defined in Claim 9 which expresses said polypeptide, or a variant, fragment, derivative or fusion thereof or fusion of a said variant or fragment or derivative and isolating said polypeptide or a variant, fragment, derivative or fusion thereof or fusion of a said variant, or fragment or derivative.

11. A polypeptide, or a variant, fragment, derivative or fusion thereof or fusion of a said variant or fragment or derivative obtainable by the method of Claim 10.

12. An antibody reactive towards a polypeptide as defined in any one of Claims 1 to 4 and 11.

13. Antibody reactive towards a polypeptide or a variant or fragment or derivative thereof according to Claim 12 wherein the antibody does

not react substantially with another stress-activated protein kinase kinase.

14. A method of identifying a compound that modulates the activity of a polypeptide as defined in Claim 1, the method comprising contacting a compound with the said polypeptide or a suitable variant, fragment, derivative or fusion thereof or a fusion of a variant, fragment or derivative thereof and determining whether the activity of the said polypeptide is changed compared to the activity of the said protein kinase or said variant, fragment, derivative or fusion thereof or a fusion of a variant, fragment or derivative thereof in the absence of said compound.

15. A method according to Claim 14 in which the activity is decreased.

16. A method according to Claim 14 in which the activity is increased.

17. A method of identifying a compound which binds to SAPK1 (or other substrate of the polypeptide as defined in Claim 1) and enhances or prevents its activation by the polypeptide as defined in Claim 1, the method comprising determining whether a compound enhances or prevents the interaction of SAPK1 (or other substrate of the polypeptide as defined in Claim 1) or a suitable fragment, variant, derivative or fusion thereof or a suitable fusion of a fragment, variant or derivative with the polypeptide as defined in Claim 1 or determining whether the compound substantially blocks activation of SAPK1 (or other substrate of the polypeptide as defined in Claim 1) or a suitable fragment, variant, derivative or fusion thereof or a

suitable fusion of a fragment, variant or derivative by the polypeptide as defined in claim 1.

18. A method of identifying a compound which blocks the activation of a polypeptide as defined in Claim 1 by an interacting polypeptide, such as MEKK, the method comprising determining whether a compound enhances or disrupts the interaction between (a) a polypeptide as defined in the first aspect of the invention or a suitable fragment, variant, derivative or fusion thereof or a suitable fusion of a fragment, variant or derivative and (b) the interacting polypeptide, such as MEKK, or a suitable variant, derivative, fragment or fusion thereof or a suitable fusion of a variant, derivative or fragment, or determining whether the compound substantially blocks activation of the said polypeptide or a suitable variant, fragment, derivative or fusion thereof, or a fusion of a said fragment, derivative or fusion by the interacting polypeptide, such as MEKK, or a suitable variant, derivative, fragment or fusion thereof.

19. The use of MEKK for the activation of the polypeptide as defined in Claim 1.

20. A method of identifying a polypeptide that interacts with a polypeptide as defined in Claim 1, the method comprising 1) contacting a) the polypeptide as defined in Claim 1 with b) a composition that may contain such an interacting polypeptide, 2) detecting the presence of a complex containing the polypeptide as defined in Claim 1 and an interacting polypeptide, and optionally 3)

identifying any interacting polypeptide bound to the said polypeptide as defined in Claim 1.

21. A polypeptide identifiable by the method of Claim 20.

22. A method of identifying a compound which blocks the activation of a polypeptide as defined in Claim 1 by a polypeptide as defined in Claim 21 the method comprising determining whether a compound enhances or disrupts the interaction between (a) a polypeptide as defined in Claim 1 and (b) said polypeptide as defined in Claim 21 or a suitable variant, derivative, fragment or fusion thereof or a suitable fusion of a variant, derivative or fragment, or determining whether the compound substantially blocks activation of the said polypeptide as defined in Claim 1 by said polypeptide as defined in Claim 21 or a suitable variant, derivative, fragment or fusion thereof.

23. A compound identifiable by the method of any one of Claims 14-18, 20, 22.

24. A compound according to Claim 23 for use in medicine.

25. A method of treating a patient with an inflammatory or ischaemic disease the method comprising administering to the patient an effective amount of a compound according to Claim 23.

26. Use of a polypeptide as defined in Claim 1 in a screening assay for compounds which modulate the activity of the said polypeptide or which modulate the activation of said polypeptide.

27. A kit of parts comprising a polypeptide as defined in Claim 1 and a means for carrying out the method as defined in any one of Claims 14-18, 20, 22.

28. Any novel stress-activated protein kinase as herein disclosed.

```
TGCTCCTGCCCCGTCCCAACGAGCAGCCCTGCAGCTCCCGCTGGCCAACGATGGGGGCAG      60

CCGCTCGCCATCCTCAGAGAGCTCCCCGCAGCACCCCACGCCCCCCGCCCGGCCCCGCCA     120

                                          M  E  S  I  E  I  D      7
CATGCTGGGGCTCCCGTCAACCCTGTTCACACCCCGCAGCATGGAGAGCATTGAGATTGA     180

 Q  K  L  Q  E  I  M  K  Q  T  G  Y  L  T  I  G  G  Q  R  Y     27
CCAGAAGCTGCAGGAGATCATGAAGCAGACGGGCTACCTGACCATCGGGGGCCAGCGCTA     240

 Q  A  E  I  N  D  L  E  N  L  G  E  M  G  S  G  T  C  G  Q     47
CCAGGCAGAAATCAACGACCTGGAGAACTTGGGCGAGATGGGCAGCGGCACCTGCGGCCA     300

 V  W  K  M  R  F  R  K  T  G  H  V  I  A  V  K  Q  M  R  R     67
GGTGTGGAAGATGCGCTTCCGGAAGACCGGCCACGTCATTGCCGTTAAGCAAATGCGGCG     360

 S  G  N  K  E  E  N  K  R  I  L  M  D  L  D  V  V  L  K  S     87
CTCCGGGAACAAGGAGGAGAACAAGCGCATCCTCATGGACCTGGATGTGGTGCTGAAGAG     420

 H  D  C  P  Y  I  V  Q  C  F  G  T  F  I  T  N  T  D  V  F    107
CCACGACTGCCCCTACATCGTGCAGTGCTTTGGGACGTTCATCACCAACACGGACGTCTT     480

 I  A  M  E  L  M  G  T  C  A  E  K  L  K  K  R  M  Q  G  P    127
CATCGCCATGGAGCTCATGGGCACCTGCGCTGAGAAGCTCAAGAAGCGGATGCAGGGCCC     540

 I  P  E  R  I  L  G  K  M  T  V  A  I  V  K  A  L  Y  Y  L    147
CATCCCCGAGCGCATTCTGGGCAAGATGACAGTGGCGATTGTGAAGGCGCTGTACTACCT     600

 K  E  K  H  G  V  I  H  R  D  V  K  P  S  N  I  L  L  D  E    167
GAAGGAGAAGCACGGTGTCATCCACCGCGACGTCAAGCCCTCCAACATCCTGCTGGACGA     660
```

Figure 1
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```
 R  G  Q  I  K  L  C  D  F  G  I  S  G  R  L  V  D  S  K  A    187
GCGGGGCCAGATCAAGCTCTGCGACTTCGGCATCAGCGGCCGCCTGGTGGACTCCAAAGC      720

 K  T  R  S  A  G  C  A  A  Y  M  A  P  E  R  I  D  P  P  D    207
CAAGACGCGGAGCGCCGGCTGTGCCGCCTACATGGCACCCGAGCGCATTGACCCCCCAGA      780

 P  T  K  P  D  Y  D  I  R  A  D  V  W  S  L  G  I  S  L  V    227
CCCCACCAAGCCGGACTATGACATCCGGGCCGACGTATGGAGCCTGGGCATCTCGTTGGT      840

 E  L  A  T  G  Q  F  P  Y  K  N  C  K  T  D  F  E  V  L  T    247
GGAGCTGGCAACAGGACAGTTTCCCTACAAGAACTGCAAGACGGACTTTGAGGTCCTCAC      900

 K  V  L  Q  E  E  P  P  L  L  P  G  H  M  G  F  S  G  D  F    267
CAAAGTCCTACAGGAAGAGCCCCCGCTTCTGCCCGGACACATGGGCTTCTCGGGGGACTT      960

 Q  S  F  V  K  D  C  L  T  K  D  H  R  K  R  P  K  Y  N  K    287
CCAGTCCTTCGTCAAAGACTGCCTTACTAAAGATCACAGGAAGAGACCAAAGTATAATAA     1020

 L  L  E  H  S  F  I  K  R  Y  E  T  L  E  V  D  V  A  S  W    307
GCTACTTGAACACAGCTTCATCAAGCGCTACGAGACGCTGGAGGTGGACGTGGCGTCCTG     1080

 F  K  D  V  M  A  K  T  E  S  P  R  T  S  G  V  L  S  Q  P    327
GTTCAAGGATGTCATGGCGAAGACTGAGTCACCGCGGACTAGCGGCGTCCTGAGCCAGCC     1140

 H  L  P  F  F  R  *                                            333
CCACCTGCCCTTCTTCAGGTAGCTGCTTGGCGGCGGCCAGCCCCACAGGGGGCCAGGGGC     1200

CGG                                                              1203
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Figure 1

2/8

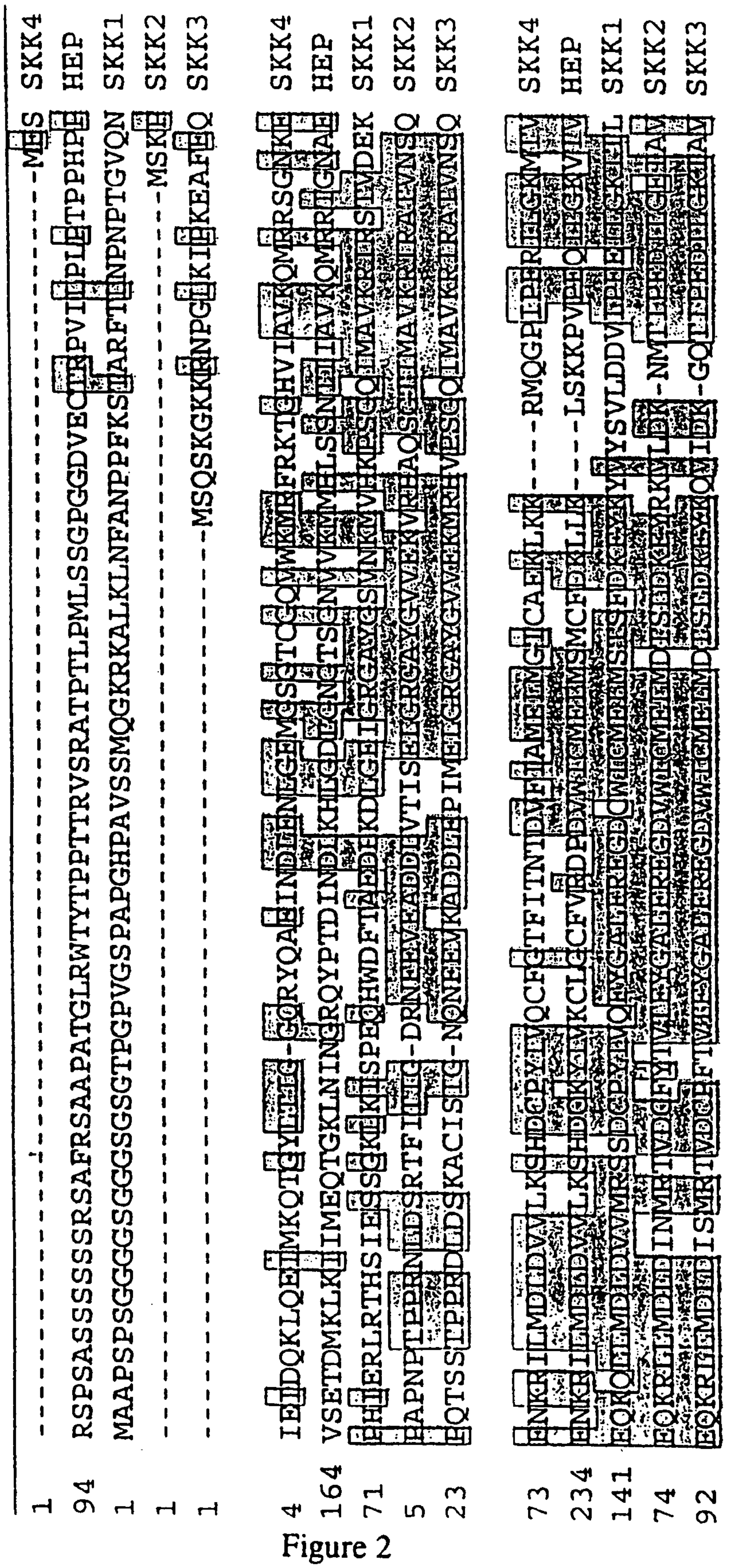

Figure 2

3/8

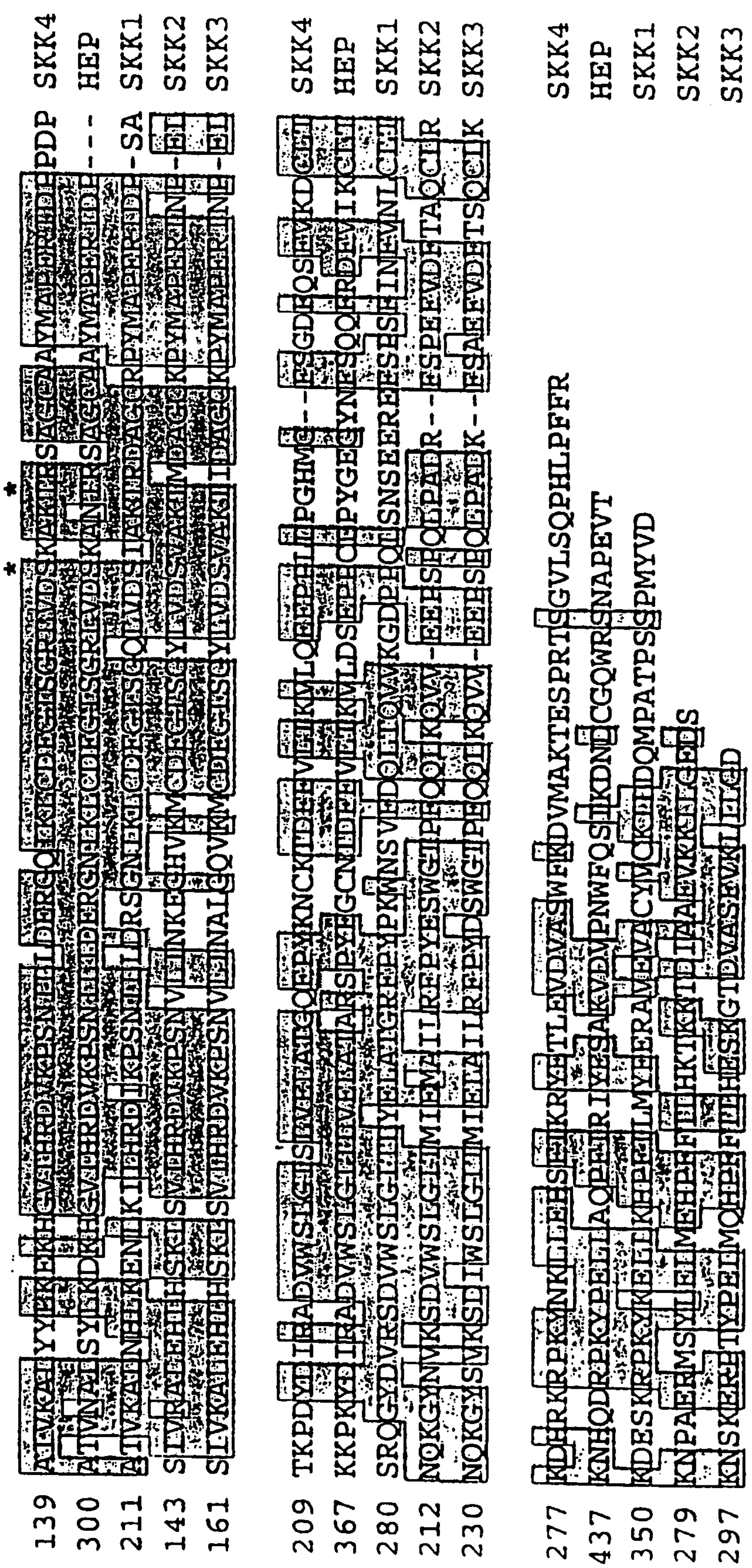

Figure 2

4/8

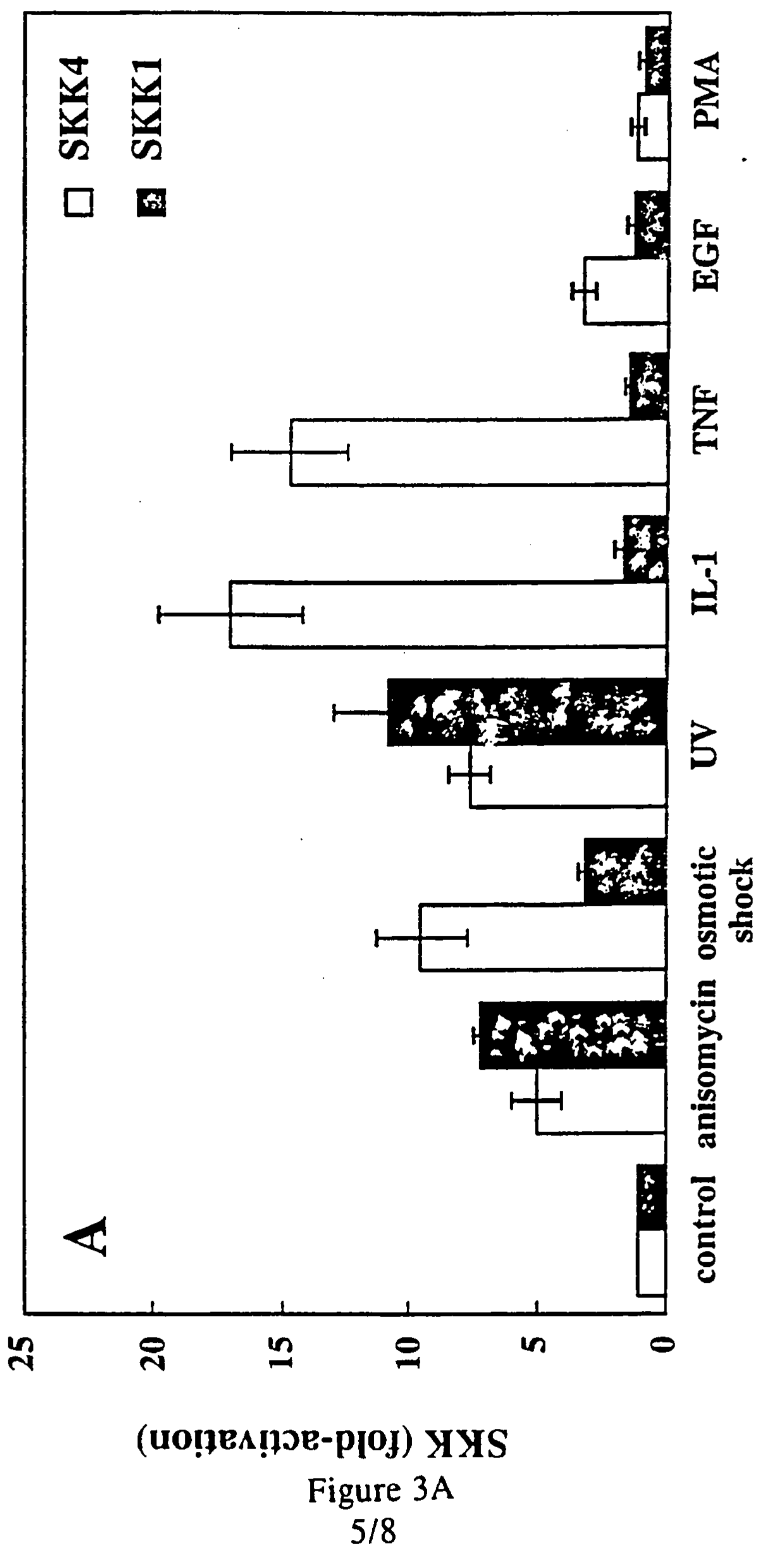
A
SKK4
SKK1
control
anisomycin
osmotic shock
UV
IL-1
TNF
EGF
PMA
0
5
10
15
20
25
SKK (fold-activation)

Figure 3A

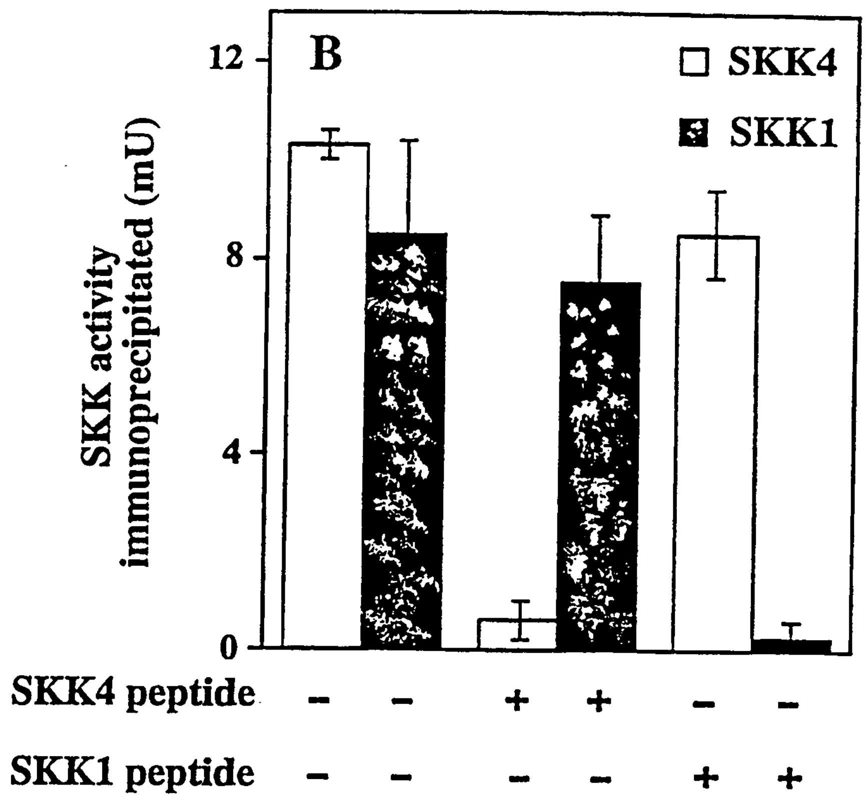
B
SKK4
SKK1
SKK activity immunoprecipitated (mU)
12
8
4
0
SKK4 peptide – – + + – –
SKK1 peptide – – – – + +

Figure 3B
6/8

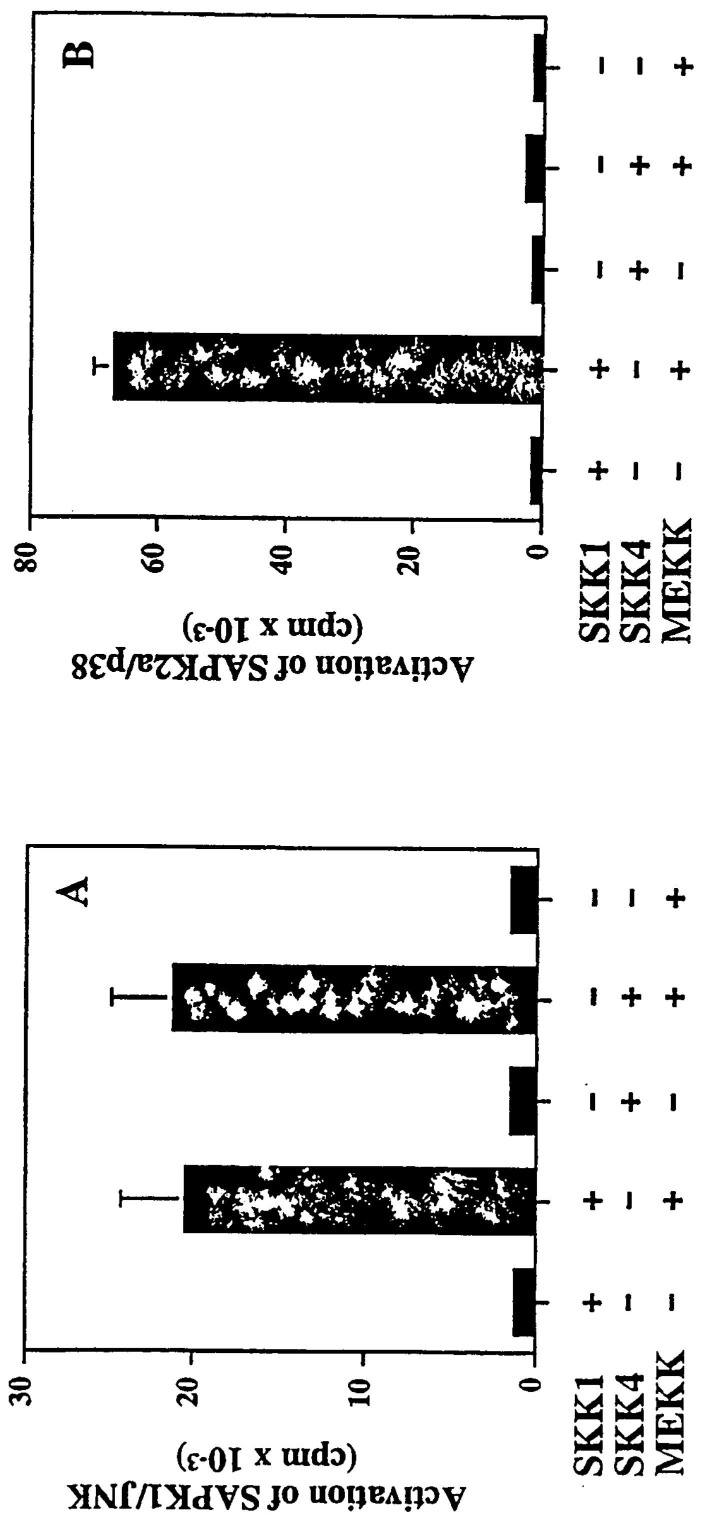
A
Activation of SAPK1/JNK
(cpm x 10-3)
0
10
20
30
SKK1
SKK4
MEKK
B
Activation of SAPK2a/p38
(cpm x 10-3)
0
20
40
60
80
SKK1
SKK4
MEKK

Figure 4

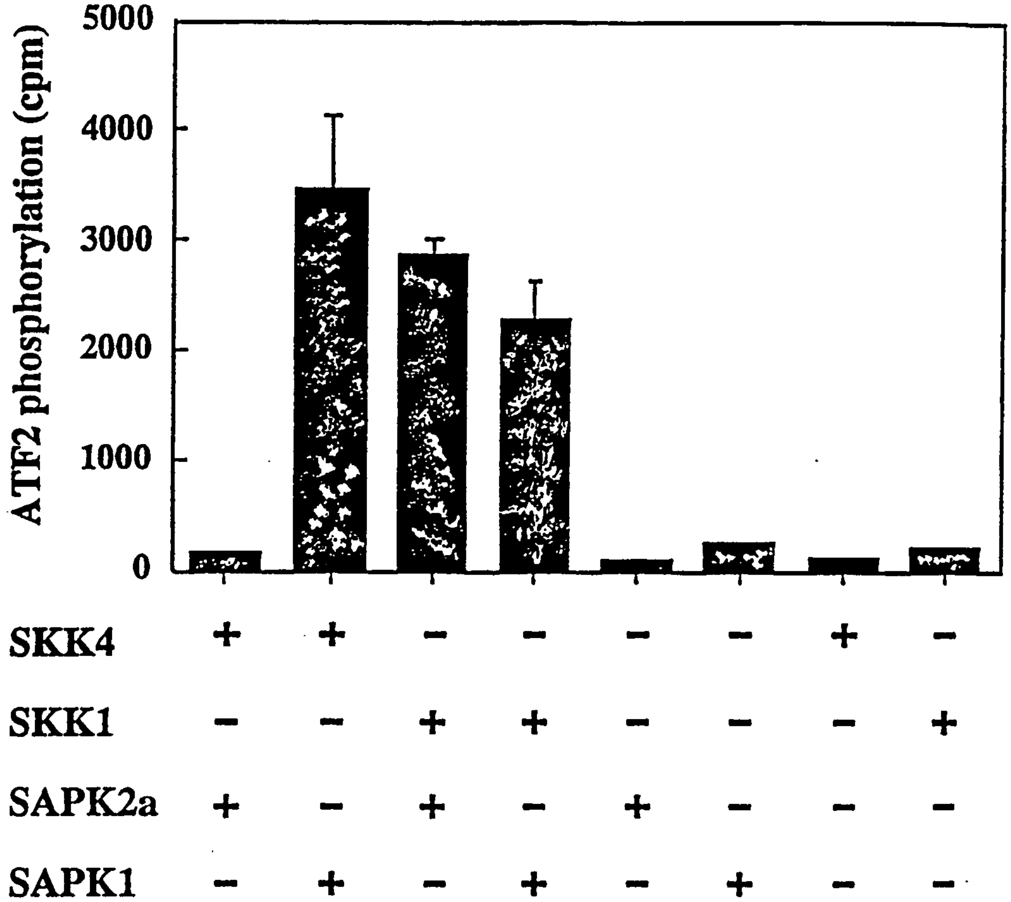
ATF2 phosphorylation (cpm)
5000
4000
3000
2000
1000
0
SKK4 + + − − − − + −
SKK1 − − + + − − − +
SAPK2a + − + − + − − −
SAPK1 − + − + − + − −

Figure 5
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SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT:
(A) NAME: Medical Research Council
(B) STREET: 20 Park Crescent
(C) CITY: London
(E) COUNTRY: United Kingdom
(F) POSTAL CODE (ZIP): W1N 4AL

(ii) TITLE OF INVENTION: Polypeptides, polynucleotides and uses thereof

(iii) NUMBER OF SEQUENCES: 6

(iv) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30 (EPO)

(2) INFORMATION FOR SEQ ID NO: 1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 333 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 1:

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Met Glu Ser Ile Glu Ile Asp Gln Lys Leu Gln Glu Ile Met Lys Gln
1               5                   10                  15

Thr Gly Tyr Leu Thr Ile Gly Gly Gln Arg Tyr Gln Ala Glu Ile Asn
            20                  25                  30

Asp Leu Glu Asn Leu Gly Glu Met Gly Ser Gly Thr Cys Gly Gln Val
        35                  40                  45

Trp Lys Met Arg Phe Arg Lys Thr Gly His Val Ile Ala Val Lys Gln
    50                  55                  60

Met Arg Arg Ser Gly Asn Lys Glu Glu Asn Lys Arg Ile Leu Met Asp
65                  70                  75                  80

Leu Asp Val Val Leu Lys Ser His Asp Cys Pro Tyr Ile Val Gln Cys
                85                  90                  95

Phe Gly Thr Phe Ile Thr Asn Thr Asp Val Phe Ile Ala Met Glu Leu
            100                 105                 110

Met Gly Thr Cys Ala Glu Lys Leu Lys Lys Arg Met Gln Gly Pro Ile
        115                 120                 125

Pro Glu Arg Ile Leu Gly Lys Met Thr Val Ala Ile Val Lys Ala Leu
    130                 135                 140

Tyr Tyr Leu Lys Glu Lys His Gly Val Ile His Arg Asp Val Lys Pro
```

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145                 150                 155                 160

Ser Asn Ile Leu Leu Asp Glu Arg Gly Gln Ile Lys Leu Cys Asp Phe
                165                 170                 175

Gly Ile Ser Gly Arg Leu Val Asp Ser Lys Ala Lys Thr Arg Ser Ala
            180                 185                 190

Gly Cys Ala Ala Tyr Met Ala Pro Glu Arg Ile Asp Pro Pro Asp Pro
        195                 200                 205

Thr Lys Pro Asp Tyr Asp Ile Arg Ala Asp Val Trp Ser Leu Gly Ile
    210                 215                 220

Ser Leu Val Glu Leu Ala Thr Gly Gln Phe Pro Tyr Lys Asn Cys Lys
225                 230                 235                 240

Thr Asp Phe Glu Val Leu Thr Lys Val Leu Gln Glu Glu Pro Pro Leu
                245                 250                 255

Leu Pro Gly His Met Gly Phe Ser Gly Asp Phe Gln Ser Phe Val Lys
            260                 265                 270

Asp Cys Leu Thr Lys Asp His Arg Lys Arg Pro Lys Tyr Asn Lys Leu
        275                 280                 285

Leu Glu His Ser Phe Ile Lys Arg Tyr Glu Thr Leu Glu Val Asp Val
    290                 295                 300

Ala Ser Trp Phe Lys Asp Val Met Ala Lys Thr Glu Ser Pro Arg Thr
305                 310                 315                 320

Ser Gly Val Leu Ser Gln Pro His Leu Pro Phe Phe Arg
                325                 330
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(2) INFORMATION FOR SEQ ID NO: 2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 999 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 2:

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ATGGAGAGCA TTGAGATTGA CCAGAAGCTG CAGGAGATCA TGAAGCAGAC GGGCTACCTG     60

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GGCAGCGGCA CCTGCGGCCA GGTGTGGAAG ATGCGCTTCC GGAAGACCGG CCACGTCATT    180

GCCGTTAAGC AAATGCGGCG CTCCGGGAAC AAGGAGGAGA ACAAGCGCAT CCTCATGGAC    240

CTGGATGTGG TGCTGAAGAG CCACGACTGC CCCTACATCG TGCAGTGCTT TGGGACGTTC    300

ATCACCAACA CGGACGTCTT CATCGCCATG GAGCTCATGG GCACCTGCGC TGAGAAGCTC    360

AAGAAGCGGA TGCAGGGCCC CATCCCCGAG CGCATTCTGG GCAAGATGAC AGTGGCGATT    420

GTGAAGGCGC TGTACTACCT GAAGGAGAAG CACGGTGTCA TCCACCGCGA CGTCAAGCCC    480

TCCAACATCC TGCTGGACGA GCGGGGCCAG ATCAAGCTCT GCGACTTCGG CATCAGCGGC    540
```

CGCCTGGTGG ACTCCAAAGC CAAGACGCGG AGCGCCGGCT GTGCCGCCTA CATGGCACCC 600

GAGCGCATTG ACCCCCCAGA CCCCACCAAG CCGGACTATG ACATCCGGGC CGACGTATGG 660

AGCCTGGGCA TCTCGTTGGT GGAGCTGGCA ACAGGACAGT TTCCCTACAA GAACTGCAAG 720

ACGGACTTTG AGGTCCTCAC CAAAGTCCTA CAGGAAGAGC CCCCGCTTCT GCCCGGACAC 780

ATGGGCTTCT CGGGGGACTT CCAGTCCTTC GTCAAAGACT GCCTTACTAA AGATCACAGG 840

AAGAGACCAA AGTATAATAA GCTACTTGAA CACAGCTTCA TCAAGCGCTA CGAGACGCTG 900

GAGGTGGACG TGGCGTCCTG GTTCAAGGAT GTCATGGCGA AGACTGAGTC ACCGCGGACT 960

AGCGGCGTCC TGAGCCAGCC CCACCTGCCC TTCTTCAGG 999

(2) INFORMATION FOR SEQ ID NO: 3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 30 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "pcr primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 3:

GACGGATCCG ACCCCACCAA GCCGGACTTT 30

(2) INFORMATION FOR SEQ ID NO: 4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 31 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "pcr primer"

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 4:

GGCCAAGCTT GTCTTTGACG AAGGACTGGA A 31

(2) INFORMATION FOR SEQ ID NO: 5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 21 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(v) FRAGMENT TYPE: internal

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 5:

```
Thr Thr Tyr Ala Asp Phe Ile Ala Ser His Gly Arg Thr Gly Arg Arg
1               5                   10                  15

Asn Ala Ile His Asp
            20
```

(2) INFORMATION FOR SEQ ID NO: 6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1203 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA to mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO: 6:

```
TGCTCCTGCC CCGTCCCAAC GAGCAGCCCT GCAGCTCCCG CTGGCCAACG ATGGGGGCAG      60
CCGCTCGCCA TCCTCAGAGA GCTCCCCGCA GCACCCCACG CCCCCCGCCC GGCCCCGCCA     120
CATGCTGGGG CTCCCGTCAA CCCTGTTCAC ACCCCGCAGC ATGGAGAGCA TTGAGATTGA     180
CCAGAAGCTG CAGGAGATCA TGAAGCAGAC GGGCTACCTG ACCATCGGGG GCCAGCGCTA     240
CCAGGCAGAA ATCAACGACC TGGAGAACTT GGGCGAGATG GGCAGCGGCA CCTGCGGCCA     300
GGTGTGGAAG ATGCGCTTCC GGAAGACCGG CCACGTCATT GCCGTTAAGC AAATGCGGCG     360
CTCCGGGAAC AAGGAGGAGA ACAAGCGCAT CCTCATGGAC CTGGATGTGG TGCTGAAGAG     420
CCACGACTGC CCCTACATCG TGCAGTGCTT TGGGACGTTC ATCACCAACA CGGACGTCTT     480
CATCGCCATG GAGCTCATGG GCACCTGCGC TGAGAAGCTC AAGAAGCGGA TGCAGGGCCC     540
CATCCCCGAG CGCATTCTGG GCAAGATGAC AGTGGCGATT GTGAAGGCGC TGTACTACCT     600
GAAGGAGAAG CACGGTGTCA TCCACCGCGA CGTCAAGCCC TCCAACATCC TGCTGGACGA     660
GCGGGGCCAG ATCAAGCTCT GCGACTTCGG CATCAGCGGC CGCCTGGTGG ACTCCAAAGC     720
CAAGACGCGG AGCGCCGGCT GTGCCGCCTA CATGGCACCC GAGCGCATTG ACCCCCCAGA     780
CCCCACCAAG CCGGACTATG ACATCCGGGC CGACGTATGG AGCCTGGGCA TCTCGTTGGT     840
GGAGCTGGCA ACAGGACAGT TTCCCTACAA GAACTGCAAG ACGGACTTTG AGGTCCTCAC     900
CAAAGTCCTA CAGGAAGAGC CCCCGCTTCT GCCCGGACAC ATGGGCTTCT CGGGGGACTT     960
CCAGTCCTTC GTCAAAGACT GCCTTACTAA AGATCACAGG AAGAGACCAA AGTATAATAA    1020
GCTACTTGAA CACAGCTTCA TCAAGCGCTA CGAGACGCTG GAGGTGGACG TGGCGTCCTG    1080
```

GTTCAAGGAT GTCATGGCGA AGACTGAGTC ACCGCGGACT AGCGGCGTCC TGAGCCAGCC 1140

CCACCTGCCC TTCTTCAGGT AGCTGCTTGG CGGCGGCCAG CCCCACAGGG GGCCAGGGGC 1200

CGG 1203

INTERNATIONAL SEARCH REPORT

International Application No
PCT/GB 98/02475

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12N15/54 C12N9/12 C07K16/40 G01N33/573 A61K38/17
C12N5/10

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12N C07K G01N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	TOURNIER C. ET AL.: "Mitogen-activated protein kinase kinase 7 is an activator of the c-Jun NH2-terminal kinase." PROC. NATL. ACAD. SCI. USA, vol. 94, July 1997, pages 7337-7342, XP002085856 cited in the application	1-13,28
Y	see the whole document, especially page 7338, right column, 3rd paragraph ---	14-18, 20-27
Y	WO 96 36642 A (DERIJARD BENOIT ;RAINGEAUD JOEL (FR); DAVIS ROGER J (US); GUPTA SH) 21 November 1996 see page 8, line 21 - page 11, line 24 ---	14-18, 20-27
	-/--	

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 November 1998	09/12/1998

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Mandl, B

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	EMBL database entry HS962239; accession number H85962; 22. Novemebr 1995 Hillier et al.:' The WashU-Merck EST project.' XP002085863 cited in the application see abstract ---	1-11
X	EMBL database entry HSAA14581; accession number AA194047; 24. January 1997; Hillier et al.: 'The WashU-Merck EST project.' XP002085864 cited in the application see abstract ---	1-11
X	EMBL database entry HSAA52650; accession number AA252650; 15. March 1997; Strausberg R.: 'National Cancer Institute, Cancer Genome Anatomy Project.' XP002085865 cited in the application see abstract ---	1-11
A	MEIER R. ET AL.: "Cellular stresses and cytokines activate multiple mitogen-activated protein kinase kinase homologues in PC12 and KB cells." EUROPEAN JOURNAL OF BIOCHEMISTRY, vol. 236, 1996, pages 796-805, XP002085857 cited in the application see the whole document ---	1-28
A	MORIGUCHI T. ET AL.: "Evidence for multiple activators for stress-activated protein kinases/c-Jun amino-terminal kinases." JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 270, no. 22, 1995, pages 12969-12972, XP002085858 cited in the application ---	1-28
A	YAN M. ET AL.: "Activation of stress-activated protein kinase by MEKK1 phosphorylation of its activator SEK1." NATURE, vol. 372, 1994, pages 798-800, XP002085859 cited in the application see abstract; figure 4 --- -/--	19

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
P,X	LAWLER, SEAN ET AL: "SKK4, a novel activator of stress -activated protein kinase -1 (SAPK1 /JNK)" FEBS LETTERS, vol. 414, no. 1, 1 September 1997, pages 153-158, XP002085860 see the whole document	1-13,19, 28
P,X	FOLTZ I. N. ET AL.: "Human mitogen-activated protein kinase kinase 7 (MKK7) is a highly conserved c-Jun N-terminal kinase/stress-activated protein kinase (JNK/SAPK) activated by environmental stresses and physiological stimuli. " JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 273, no. 15, April 1998, pages 9344-9351, XP002085861 see the whole document	1-13,19, 28
P,X	YANG J. ET AL.: "Molecular cloning and characterization of a human protein kinase that specifically activates c-Jun N-terminal kinase." GENE, vol. 212, 28 May 1998, pages 95-102, XP002085862 see the whole document	1-13,28

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/GB 98/02475

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claim 25
is directed to a method of treatment of the human/animal body, the search has been carried out and based on the alleged effects of the compound/composition.

2. [ ] Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/GB 98/02475

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9636642 A	21-11-1996	US 5804427 A	08-09-1998
		US 5736381 A	07-04-1998
		AU 4904696 A	29-11-1996
		CA 2219487 A	21-11-1996
		EP 0830374 A	25-03-1998